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(54) Title: MURE PROTEIN AND GENE OF *PSEUDOMONAS AERUGINOSA*

(57) Abstract: This invention provides isolated polynucleotides that encode the MurE protein of *Pseudomonas aeruginosa*. Purified and isolated MurE recombinant proteins are also provided. Nucleic acid sequences which encode functionally active MurE proteins are described. Assays for the identification of modulators of the expression of murE and inhibitors of the activity of *MurE*, are also provided.

WO 01/19843 A1

TITLE OF THE INVENTION

MURE PROTEIN AND GENE OF *PSEUDOMONAS AERUGINOSA*

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/154,117, filed September 15, 1999, the contents of which are incorporated herein by reference in their entirety.

STATEMENT REGARDING FEDERALLY-SPONSORED R&D

Not applicable.

REFERENCE TO MICROFICHE APPENDIX

Not applicable.

FIELD OF THE INVENTION

This invention relates to the genes and enzymes involved in cell wall synthesis in bacteria, and particularly to the inhibition of such enzymes.

BACKGROUND OF THE INVENTION

The bacterial cell wall plays a pivotal role in maintaing the cell shape and integrity. It is also a dynamic structure that permits cell expansion and division. Compounds that interfere with the biosynthesis or integrity of this compartment are bactericidal. The bacterial cell wall is comprised of peptidoglycan (PG), a network of polysaccharide chains cross-linked by short peptides (Rogers, H. J., H. R. Perkins, and J. B. Ward, 1980, Biosynthesis of peptidoglycan. p. 239-297. In Microbial cell walls and membranes. Chapman & Hall Ltd. London). Biosynthesis of PG can be divided into three functional stages. Stage I reactions takes place in the cytoplasm and includes the activities of MurA-through MurF leading to the synthesis of PG nucleotide precursors. Stage II include membrane reactions catalyzed by MraY and MurG. Stage III are mediated by Penicillin-binding proteins in the periplasmic space (Bugg, T. D. H. and Walsh, C. T. 1992. Intracellular steps of bacterial cell wall peptidoglycan biosynthesis: enzymology, antibiotics, and antibiotic resistance. Nat. Prod. Rep. 9, 199-215)

A basic repeating structure of PG consists of the disaccharides *N*-acetylglucosamine (GlucNAc) and *N*-acetylmuramic acid (MurNAc). The GlucNAc and the MurNAc disaccharides are cross-linked by b-1,4-glycosidic linkages. The MurNAc is linked to a side chain of tetra- or pentapeptide consisting of L-alanine, D-glutamate, m-diaminopimelic acid and ending in either D-alanine or D-alanyl-D-alanine. Only the tetrapeptide side chain, but not the pentapeptide chain participates in cross-linking.

The successive addition of the amino acids in the above peptide chains is catalyzed by four different ligases during the cytoplasmic steps in the bisynthesis of PG. The genes encoding these enzymes are essential for growth validating each of them as antibiotic target. Several lines of evidence support the notion that these ligases are potential target for new antibiotics. These enzymes are highly specific and present in both Gram(-) and Gram (+) bacteria (Bugg, T. D. H. and Walsh, C. T. 1992.).

MurE, the *meso*-diaminopimelic acid-adding enzyme is an ATP-dependent amino acid ligase that is responsible for the formation of UDP-MurNAc-L-ala-D-Glu-*m*-Dap (Michaud C, D. Mengin-Lecreulx, J. van Heijenoort, D. Blanot . 1990. Over-production, purification and properties of the uridine-diphosphate-N-acetylmuramoyl-L-alanyl-D-glutamate:meso-2,6-diaminopimelate ligase from *Escherichia coli.* Eur J Biochem 194(3):853-861). Inhibitors of this enzyme could lead to new antibiotics for treating infections with Gram (-ve) bacteria such as *Pseudomonas aeruginosa.*

SUMMARY OF THE INVENTION

Polynucleotides and polypeptides of *Pseudomonas aeruginosa* MurE, an enzyme involved in bacterial cell wall biosynthesis are provided. The recombinant MurE enzyme is catalytically active in ATP-dependent *meso*-Diaminopimelic acid (*m*-Dap) addition reactions. The enzyme is used in *in vitro* assays to screen for antibacterial compounds that target cell wall biosynthesis. The invention includes the polynucleotides, proteins encoded by the polynucleotides, and host cells expressing the recombinant enzyme, probes and primers, and the use of these molecules in assays.

An aspect of this invention is a polynucleotide having a sequence encoding a *Pseudomonas aeruginosa* MurE protein, or a complementary sequence. In a particular embodiment the encoded protein has a sequence corresponding to SEQ ID

NO:2. In other embodiments, the encoded protein can be a naturally occurring mutant or polymorphic form of the protein. In preferred embodiments the polynucleotide can be DNA, RNA or a mixture of both, and can be single or double stranded. In particular embodiments, the polynucelotide is comprised of natural, non-natural or modified nucleotides. In some embodiments, the internucleotide linkages are linkages that occur in nature. In other embodiments, the internucleotide linkages can be non-natural linkages or a mixture of natural and non-natural linkages. In a most preferred embodiment, the polynucleotide has a sequence shown in SEQ ID NO:1.

An aspect of this invention is a polynucleotide having a sequence of at least about 25 contiguous nucleotides that is specific for a naturally occurring polynucleotide encoding a *Pseudomonas aeruginosa* MurE protein. In particular preferred embodiments, the polynucleotides of this aspect are useful as probes for the specific detection of the presence of a polynucleotide encoding a *Pseudomonas aeruginosa* MurE protein. In other particular embodiments, the polynucleotides of this aspect are useful as primers for use in nucleic acid amplification based assays for the specific detection of the presence of a polynucleotide encoding a *Pseudomonas aeruginosa* MurE protein. In preferred embodiments, the polynucleotides of this aspect can have additional components including, but not limited to, compounds, isotopes, proteins or sequences for the detection of the probe or primer.

An aspect of this invention is an expression vector including a polynucleotide encoding a *Pseudomonas aeruginosa* MurE protein, or a complementary sequence, and regulatory regions. In a particular embodiment the encoded protein has a sequence corresponding to SEQ ID NO:2. In particular embodiments, the vector can have any of a variety of regulatory regions known and used in the art as appropriate for the types of host cells the vector can be used in. In a most preferred embodiment, the vector has regulatory regions appropriate for the expression of the encoded protein in gram-negative prokaryotic host cells. In other embodiments, the vector has regulatory regions appropriate for expression of the encoded protein in gram-positive host cells, yeasts, cyanobacteria or actinomycetes. In some preferred embodiments the regulatory regions provide for inducible expression while in other preferred embodiments the regulatory regions provide for constitutive expression. Finally, according to this aspect, the expression vector can be derived from a plasmid, phage, virus or a combination thereof.

An aspect of this invention is host cell comprising an expression vector including a polynucleotide encoding a *Pseudomonas aeruginosa* MurE protein, or a

complementary sequence, and regulatory regions. In a particular embodiment the encoded protein has a sequence corresponding to SEQ ID NO:2. In preferred embodiments, the host cell is a yeast, gram-positive bacterium, cyanobacterium or actinomycete. In a most preferred embodiment, the host cell is a gram-negative bacterium.

An aspect of this invention is a process for expressing a MurE protein of *P. aeruginosa* in a host cell. In this aspect a host cell is transformed or transfected with an expression vector including a polynucleotide encoding a *Pseudomonas aeruginosa* MurE protein, or a complementary sequence. According to this aspect, the host cell is cultured under conditions conducive to the expression of the encoded MurE protein. In particular embodiments the expression is inducible or constitutive. In a particular embodiment the encoded protein has a sequence corresponding to SEQ ID NO:2.

An aspect of this invention is a purified polypeptide having an amino acid sequence of SEQ ID NO:2 or the sequence of a naturally occurring mutant or polymorphic form of the protein.

An aspect of this invention is a method of determining whether a candidate compound can inhibit the activity of a *P. aeruginosa* MurE polypeptide. According to this aspect a polynucleotide encoding the polypeptide is used to construct an expression vector appropriate for a particular host cell. The host cell is transformed or transfected with the expression vector and cultured under conditions conducive to the expression of the MurE polypeptide. The cell or a preparation derived therefrom containing the MurE polypeptide is contacted with the candidate. Finally, one measures the activity of the MurE polypeptide in the presence of the candidate. If the activity is lower relative to the activity of the protein in the absence of the candidate, then the candidate is a inhibitor of the MurE polypeptide. In preferred embodiments, the polynucleotide encodes a protein having an amino acid sequence of SEQ ID NO:2 or a naturally occurring mutant of polymorphic form thereof. In other preferred embodiments, the polynucleotide has the sequence of SEQ ID NO:1. In particular embodiments, the relative activity of MurE is determined by comparing the activity of the MurE in a host cell or a preparation derived therefrom containing the MurE polypeptide. In some embodiments, the host cell is disrupted and the candidate is contacted to the released cytosol. In other embodiments, the cells can be disrupted after contacting with the candidate and before determining the activity of the MurE protein. Finally, according to this aspect the relative activity can

determined by comparison to a previously measured or expected activity value for the MurE activity in the host, or preparation, under the conditions. However, in preferred embodiments, the relative activity is determined by measuring the activity of the MurE in a control cell, or preparation, that was not contacted with a candidate compound. In particular embodiments, the host cell is a pseudomonad and the protein inhibited is the MurE produced by the pseudomonad.

An aspect of this invention is a compound that is an inhibitor of a *P. aeruginosa* MurE protein an assay described herein. In preferred embodiments, the compound is an inhibitor of a *P. aeruginosa* MurE protein produced by a host cell comprising an expression vector of this invention. In most preferred embodiments, the compound is also an inhibitor of MurE protein produced by a pathogenic strain *P. aeruginosa* and also inhibits the growth of said pseudomonad.

An aspect of this invention is a pharmaceutical preparation that includes an inhibitor of *P. aeruginosa* MurE and a pharmaceutically acceptable carrier.

An aspect of this invention is a method of treatment comprising administering a inhibitor of the *P. aeruginosa* MurE to a patient. The treatment can be prophylactic or therapeutic. In preferred embodiments, the appropriate dosage for a particular patient is determined by a physician.

By "about" it is meant within 10-20% greater or lesser than particularly stated.

As used herein an "inhibitor" is a compound that interacts with and inhibits or prevents a polypeptide of MurE from catalyzing the ATP-dependent addition of *m*-Dap to the D-glutamate residue of the UDP-N-acetylmuramyl-L-alanine-D-Glutamine precursor.

As used herein a "modulator" is a compound that interacts with an aspect of cellular biochemistry to effect an increase or decrease in the amount of a polypeptide of MurE present in, at the surface or in the periplasm of a cell, or in the surrounding serum or media. The change in amount of the MurE polypeptide can be mediated by the effect of a modulator on the expression of the protein, *e.g.*, the transcription, translation, post-translational processing, translocation or folding of the protein, or by affecting a component(s) of cellular biochemistry that directly or indirectly participates in the expression of the protein. Alternatively, a modulator can act by accelerating or decelerating the turnover of the protein either by direct

interaction with the protein or by interacting with another component(s) of cellular biochemistry which directly or indirectly effects the change.

All of the references cited herein are incorporated by reference in their entirety as background material.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A & 1B. Nucleotide sequence (SEQ ID NO: 1) and the predicted amino acid sequence (SEQ ID NO:2) of *P. aeruginosa murE*. The amino acid sequence (SEQ ID NO:2) is presented in three-letter code.

FIG. 2. Production of recombinant *P. aeruginosa* MurE. Aliquots from cell lysates, either uninduced or induced with IPTG, and column-purified polypeptides were analyzed by SDS-PAGE. Lane 1, Molecular weight markers; Lane2, IPTG-induced lysate of cells (BL21(DE3)/pLysS) containing the control vector pET-15b; Lane 3, uninduced cell lysate containing the control vector pET-15b; lane 4, column-purified MurE; Lane 5 IPTG-induced lysate of cells expressing MurE; Lane 6, uninduced lysate of cells containing *murE*..

DETAILED DESCRIPTION OF THE INVENTION

This invention provides polynucleotides and polypeptides of a cell wall biosynthesis gene from *Pseudomonas aeruginosa*, referred to herein as MurE. The polynucleotides and polypeptides are used to further provide expression vectors, host cells comprising the vectors, probes and primers, antibodies against the MurE protein and polypeptides thereof, assays for the presence or expression of MurE and assays for the identification of modulators and inhibitors of MurE.

Bacterial MurE, UDP-N-acetylmuramoyl-L-alanyl-D-glutamate:*meso*-2,6-diaminopimelate ligase, a cytoplasmic peptidoglycan biosynthetic enzyme, catalyzes the ATP-dependent addition of *m*-Dap to the D-Glutamine residue of the UDP-N-acetylmuramyl-L-alanine-D-Glutamine precursor.

The *murE* gene was cloned from *Pseudomonas aeruginosa*. Sequence analysis of the *P. aeruginosa murE* gene revealed an open reading frame of 487 amino acids. The deduced amino acid sequence of *P. aeruginosa* MurE is homologous to MurE from *Escherichia coli* and other bacteria . Recombinant MurE protein from *P. aeruginosa* was over-produced as His-tagged fusion protein in *Escherichia coli* host cellsand the enzyme was purified to apparent homogeneity. The

recombinant enzyme catalyzed the ATP-dependent addition of diaminopimelic acid to a precursor sugar peptide.

Polynucleotides

Nucleic acids encoding *murE* from *Pseudomonas aeruginosa* are useful in the expression and production of the *P. aeruginosa* MurE protein. The nucleic acids are also useful in providing probes for detecting the presence of *P. aeruginosa mur E.*

A preferred aspect of the present invention is an isolated nucleic acid encoding a MurE protein of *Pseudomonas aeruginosa*. A preferred embodiment is a nucleic acid having the sequence disclosed in FIG. 1, SEQ ID NO:1 and disclosed as follows:

ATGCCTATGA GCCTGAGCCA ACTGTTTCCC CAGGCCGAGC GCGATCTGCT

GATCCGCGAG CTGACCCTGG ATAGCCACGG CGTTCGTCCG GTCGACCTGT

TCCTGACGGT TCCGGGCGGG CACCAGGATG GTCGTGCGCA CATCGCCGAT

GCCCTGACCA AGGGCGCGAC TGCCGTGGCT TACGAGGCGG AAGGCGCCGG

AGAGTTGCCG CCCAGCGATG CGCCGCTGAT CGCGGTGAAG GGGCTGGCCG

CGCAACTGTC GGCGGTCGCC GGGCGTTTCT ACGGCGAGCC GAGCCGCGGG

CTGGACCTGA TCGGCGTCAC CGGCACCAAC GGCAAGACCA GCGTCAGCCA

ACTGGTGGCC CAGGCCCTGG ATCTGCTCGG CGAGCGCTGC GGCATCGTCG

GCACCCTCGG CACCGGTTTC TACGGCGCCC TGGAGAGCGG CCGGCACACC

ACGCCGGACC CGCTCGCGGT GCAGGCCACG CTGGCCACGC TGAAGCAGGC

CGGCGCCCGC GCGGTAGCGA TGGAAGTGTC TTCCCACGGC CTCGACCAGG

GCCGCGTGGC GGCGCTCGGC TTCGATATCG CGGTGTTCAC CAATCTGTCC

CGCGACCACC TCGACTATCA CGGTTCGATG GAAGCCTATG CCGCCGCCAA

GGCCAAGCTG TTCGCCTGGC CGGACCTGCG CTGCCGGGTG ATCAACCTGG

ACGACGATTT CGGCCGTCGA CTGGCCGGCG AGGAGCAGGA CTCGGAGCTG

ATCACCTACA GCCTCACCGA CAGCTCGGCG TTCCTCTATT GCCGCGAAGC

GCGCTTCGGC GACGCCGGCA TCGAGGCGGC GCTGGTCACT CCGCACGGCG

AGGGCCTGCT GCGCAGCCCG TTGCTCGGCC GCTTCAACCT GAGCAACCTG

CTGGCGGCGG TCGGTGCGTT GCTTGGCCTG GGTTATCCCC TGGGCGATAT

CCTCCGCACT TTGCCGCAAC TGCAGGGGCC GGTCGGCCGC ATGCAGCGCC

TGGGAGGCGG CGGCAAGCCG CTGGTGGTGG TGGACTACGC GCATACTCCC

GACGCCCTGG AAAAAGTCCT GGAGGCCCTG CGTCCGCACG CGGCCGCGCG

CCTGCTGTGC CTGTTCGGCT GCGGTGGCGA TCGCGATGCC GGCAAGCGTC

CGCTGATGGC TGCGATCGCC GAACGCCTGG CGGATGAGGT GCTGGTCACC

GACGACAACC CGCGCACCGA GGCCAGTGCG GCGATCATCG CCGATATCCG

CAAAGGCTTC GCTGCCGCTG ACAAGGTTAC CTTCCTGCCG TCGCGCGGTG

AGGCGATCGC CCATCTGATC GCTTCCGCTG CGGTGGATGA CGTGGTGCTC

CTGGCCGGCA AGGGTCACGA GGATTATCAG GAGATCGACG GCGTACGCCA

TCCGTTCTCC GACATCGAGC AGGCCGAGCG CGCCCTGGCC GCCTGGGAGG

TGCCGCATGC TTGAGCCTCT TCGCCTCAGC CAGTTGACGG TCGCGCTGG (SEQ ID NO:1)

The translation initiation and termination codons are underlined.

The isolated nucleic acid molecule of the present invention can include a ribonucleic or deoxyribonucleic acid molecule, which can be single (coding or noncoding strand) or double stranded, as well as synthetic nucleic acid, such as a synthesized, single stranded polynucleotide.

The present invention also relates to recombinant vectors and recombinant hosts, both prokaryotic and eukaryotic, which contain the substantially purified nucleic acid molecules disclosed throughout this specification.

As used herein a "polynucleotide" is a nucleic acid of more than one nucleotide. A polynucleotide can be made up of multiple polynucleotide units that are referred to by description of the unit. For example, a polynucleotide can comprise within its bounds a polynucleotide(s) having a coding sequence(s), a polynucleotide(s) that is a regulatory region(s) and/or other polynucleotide units commonly used in the art.

An "expression vector" is a polynucleotide having regulatory regions operably linked to a coding region such that, when in a host cell, the regulatory regions can direct the expression of the coding sequence. The use of expression vectors is well known in the art. Expression vectors can be used in a variety of host cells and, therefore, the regulatory regions are preferably chosen as appropriate for the particular host cell.

A "regulatory region" is a polynucleotide that can promote or enhance the initiation or termination of transcription or translation of a coding sequence. A regulatory region includes a sequence that is recognized by the RNA polymerase, ribosome, or associated transcription or translation initiation or termination factors of a host cell. Regulatory regions that direct the initiation of transcription or translation can direct constitutive or inducible expression of a coding sequence.

Polynucleotides of this invention contain full length or partial length sequences of the MurE gene sequences disclosed herein. Polynucleotides of this invention can be single or double stranded. If single stranded, the polynucleotides can be a coding, "sense," strand or a complementary, "antisense," strand. Antisense strands can be useful as modulators of the gene by interacting with RNA encoding the MurE protein. Antisense strands are preferably less than full length strands having sequences unique or specific for RNA encoding the protein.

The polynucleotides can include deoxyribonucleotides, ribonucleotides or mixtures of both. The polynucleotides can be produced by cells, in cell-free

biochemical reactions or through chemical synthesis. Non-natural or modified nucleotides, including inosine, methyl-cytosine, deaza-guanosine, etc., can be present. Natural phosphodiester internucleotide linkages can be appropriate. However, polynucleotides can have non-natural linkages between the nucleotides. Non-natural linkages are well known in the art and include, without limitation, methylphosphonates, phosphorothioates, phosphorodithionates, phosphoroamidites and phosphate ester linkages. Dephospho-linkages are also known, as bridges between nucleotides. Examples of these include siloxane, carbonate, carboxymethyl ester, acetamidate, carbamate, and thioether bridges. "Plastic DNA," having, for example, N-vinyl, methacryloxyethyl, methacrylamide or ethyleneimine internucleotide linkages, can be used. "Peptide Nucleic Acid" (PNA) is also useful and resists degradation by nucleases. These linkages can be mixed in a polynucleotide.

As used herein, "purified" and "isolated" are utilized interchangeably to stand for the proposition that the polynucleotide, protein and polypeptide, or respective fragments thereof in question have been removed from the *in vivo* environment so that they exist in a form or purity not found in nature. Purified or isolated nucleic acid molecules can be manipulated by the skilled artisan, such as but not limited to sequencing, restriction digestion, site-directed mutagenesis, and subcloning into expression vectors for a nucleic acid fragment as well as obtaining the wholly or partially purified protein or protein fragment so as to afford the opportunity to generate polyclonal antibodies, monoclonal antibodies, or perform amino acid sequencing or peptide digestion. Therefore, the nucleic acids claimed herein can be present in whole cells or in cell lysates or in a partially or substantially purified form. It is preferred that the molecule be present at a concentration at least about five-fold to ten-fold higher than that found in nature. A polynucleotide is considered substantially pure if it is obtained purified from cellular components by standard methods at a concentration of at least about 100-fold higher than that found in nature. A polynucleotide is considered essentially pure if it is obtained at a concentration of at least about 1000-fold higher than that found in nature. We most prefer polynucleotides that have been purified to homogeneity, that is, at least 10,000 - 100,000 fold. A chemically synthesized nucleic acid sequence is considered to be substantially purified when purified from its chemical precursors by the standards stated above.

Included in the present invention are assays that employ further novel polynucleotides that hybridize to *P.aeruginosa murf* sequences under stringent conditions. By way of example, and not limitation, a procedure using conditions of high stringency is as follows: Prehybridization of filters containing DNA is carried out for 2 hr. to overnight at 65°C in buffer composed of 6X SSC, 5X Denhardt's solution, and 100 μg/ml denatured salmon sperm DNA. Filters are hybridized for 12 to 48 hrs at 65°C in prehybridization mixture containing 100 μg/ml denatured salmon sperm DNA and 5-20 X 10^6 cpm of ^{32}P-labeled probe. Washing of filters is done at 37°C for 1 hr in a solution containing 2X SSC, 0.1% SDS. This is followed by a wash in 0.1X SSC, 0.1% SDS at 50°C for 45 min. before autoradiography.

Other procedures using conditions of high stringency would include either a hybridization step carried out in 5XSSC, 5X Denhardt's solution, 50% formamide at 42°C for 12 to 48 hours or a washing step carried out in 0.2X SSPE, 0.2% SDS at 65°C for 30 to 60 minutes.

Reagents mentioned in the foregoing procedures for carrying out high stringency hybridization are well known in the art. Details of the composition of these reagents can be found in, *e.g.*, Sambrook, *et al.*, 1989, Molecular Cloning: A Laboratory Manual, second edition, Cold Spring Harbor Laboratory Press. In addition to the foregoing, other conditions of high stringency which may be used are well known in the art.

Polypeptides

A preferred aspect of the present invention is a substantially purified form of the MurE protein from *Pseudomonas aeruginosa*. A preferred embodiment is a protein that has the amino acid sequence which is shown in FIG. 1, in SEQ ID NO:2 and disclosed as follows:

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MetProMetSerLeuSerGlnLeuPheProGlnAlaGluArgAspLeuLeuIleArgGlu
LeuThrLeuAspSerHisGlyValArgProValAspLeuPheLeuThrValProGlyGly
HisGlnAspGlyArgAlaHisIleAlaAspAlaLeuThrLysGlyAlaThrAlaValAla
TyrGluAlaGluGlyAlaGlyGluLeuProProSerAspAlaProLeuIleAlaValLys
GlyLeuAlaAlaGlnLeuSerAlaValAlaGlyArgPheTyrGlyGluProSerArgGly
LeuAspLeuIleGlyValThrGlyThrAsnGlyLysThrSerValSerGlnLeuValAla
GlnAlaLeuAspLeuLeuGlyGluArgCysGlyIleValGlyThrLeuGlyThrGlyPhe
TyrGlyAlaLeuGluSerGlyArgHisThrThrProAspProLeuAlaValGlnAlaThr
LeuAlaThrLeuLysGlnAlaGlyAlaArgAlaValAlaMetGluValSerSerHisGly
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LeuAspGlnGlyArgValAlaAlaLeuGlyPheAspIleAlaValPheThrAsnLeuSer
ArgAspHisLeuAspTyrHisGlySerMetGluAlaTyrAlaAlaAlaLysAlaLysLeu
PheAlaTrpProAspLeuArgCysArgValIleAsnLeuAspAspAspPheGlyArgArg
LeuAlaGlyGluGluGlnAspSerGluLeuIleThrTyrSerLeuThrAspSerSerAla
PheLeuTyrCysArgGluAlaArgPheGlyAspAlaGlyIleGluAlaAlaLeuValThr
ProHisGlyGluGlyLeuLeuArgSerProLeuLeuGlyArgPheAsnLeuSerAsnLeu
LeuAlaAlaValGlyAlaLeuLeuGlyLeuGlyTyrProLeuGlyAspIleLeuArgThr
LeuProGlnLeuGlnGlyProValGlyArgMetGlnArgLeuGlyGlyGlyGlyLysPro
LeuValValValAspTyrAlaHisThrProAspAlaLeuGluLysValLeuGluAlaLeu
ArgProHisAlaAlaAlaArgLeuLeuCysLeuPheGlyCysGlyGlyAspArgAspAla
GlyLysArgProLeuMetAlaAlaIleAlaGluArgLeuAlaAspGluValLeuValThr
AspAspAsnProArgThrGluAlaSerAlaAlaIleIleAlaAspIleArgLysGlyPhe
AlaAlaAlaAspLysValThrPheLeuProSerArgGlyGluAlaIleAlaHisLeuIle
AlaSerAlaAlaValAspAspValValLeuLeuAlaGlyLysGlyHisGluAspTyrGln
GluIleAspGlyValArgHisProPheSerAspIleGluGlnAlaGluArgAlaLeuAla
AlaTrpGluValProHisAla (SEQ ID NO:2)

The present invention also relates to biologically active fragments and mutant or polymorphic forms of MurE polypeptide sequence as set forth as SEQ ID NO: 2, including but not limited to amino acid substitutions, deletions, additions, amino terminal truncations and carboxy-terminal truncations such that these mutations provide for proteins or protein fragments of diagnostic, therapeutic or prophylactic use and would be useful for screening for modulators, and/or inhibitors of MurE function.

Using the disclosure of polynucleotide and polypeptide sequences provided herein to isolate polynucleotides encoding naturally occurring forms of MurE, one of skill in the art can determine whether such naturally occurring forms are mutant or polymorphic forms of MurE by sequence comparison. One can further determine whether the encoded protein, or fragments of any MurE protein, is biologically active by routine testing of the protein or fragment in a *in vitro* or *in vivo* assay for the biological activity of the MurE protein. For example, one can express N-terminal or C-terminal truncations, or internal additions or deletions, in host cells and test for their ability to catalyze the ATP-dependent addition of *m*-Dap to the D-Glutamine residue of the UDP-N-acetylmuramyl-L-alanine-D-Glutamine precursor.

It is known that there is a substantial amount of redundancy in the various codons which code for specific amino acids. Therefore, this invention is also

directed to those DNA sequences that encode RNA comprising alternative codons which code for the eventual translation of the identical amino acid. Therefore, the present invention discloses codon redundancy which can result in different DNA molecules encoding an identical protein. For purposes of this specification, a sequence bearing one or more replaced codons will be defined as a degenerate variation. Also included within the scope of this invention are mutations either in the DNA sequence or the translated protein which do not substantially alter the ultimate physical properties of the expressed protein. For example, substitution of valine for leucine, arginine for lysine, or asparagine for glutamine may not cause a change in functionality of the polypeptide. However, any given change can be examined for any effect on biological function by simply assaying for the ability to catalyze the ATP-dependent addition of *m*-Dap to the D-Glutamine residue of the UDP-N-acetylmuramyl-L-alanine-D-Glutamine precursor as compared to an unaltered MurE protein.

It is known that DNA sequences coding for a peptide can be altered so as to code for a peptide having properties that are different than those of the naturally occurring peptide. Methods of altering the DNA sequences include but are not limited to site directed mutagenesis. Examples of altered properties include but are not limited to changes in the affinity of an enzyme for a substrate.

As used herein, a "biologically active equivalent" or "functional derivative" of a wild-type MurE possesses a biological activity that is substantially similar to the biological activity of a wild type MurE. The term "functional derivative" is intended to include the "fragments," "mutants," "variants," "degenerate variants," "analogs," "orthologues," and "homologues" and "chemical derivatives" of a wild type MurE protein that can catalyze the ATP-dependent addition of *m*-Dap to the D-Glutamine residue of of the UDP-N-acetylmuramyl-L-alanine-D-Glutamine precursor.

The term "fragment" refers to any polypeptide subset of wild-type MurE. The term "mutant" is meant to refer to a molecule that may be substantially similar to the wild-type form but possesses distinguishing biological characteristics. Such altered characteristics include but are in no way limited to altered substrate binding, altered substrate affinity and altered sensitivity to chemical compounds affecting biological activity of the MurE or MurE functional derivative. The term "variant" refers to a molecule substantially similar in structure and function to either the entire wild-type protein or to a fragment thereof. A molecule is "substantially

similar" to a wild-type MurE-like protein if both molecules have substantially similar structures or if both molecules possess similar biological activity. Therefore, if the two molecules possess substantially similar activity, they are considered to be variants even if the exact structure of one of the molecules is not found in the other or even if the two amino acid sequences are not identical. The term "analog" refers to a molecule substantially similar in function to either the full-length MurE protein or to a biologically active fragment thereof.

As used herein in reference to a MurE gene or encoded protein, a "polymorphic" MurE is a MurE that is naturally found in the population of *Pseudomonads* at large. A polymorphic form of MurE can be encoded by a different nucleotide sequence from the particular *murE* gene disclosed herein as SEQ ID NO:1. However, because of silent mutations, a polymorphic *murE* gene can encode the same or different amino acid sequence as that disclosed herein. Further, some polymorphic forms MurE will exhibit biological characteristics that distinguish the form from wild-type MurE activity, in which case the polymorphic form is also a mutant.

A protein or fragment thereof is considered purified or isolated when it is obtained at least partially free from it's natural environment in a composition or purity not found in nature. It is preferred that the molecule be present at a concentration at least about five-fold to ten-fold higher than that found in nature. A protein or fragment thereof is considered substantially pure if it is obtained at a concentration of at least about 100-fold higher than that found in nature. A protein or fragment thereof is considered essentially pure if it is obtained at a concentration of at least about 1000-fold higher than that found in nature. We most prefer proteins that have been purified to homogeneity, that is, at least 10,000 -100,000 fold.

Probes and Primers

Polynucleotide probes comprising full length or partial sequences of SEQ ID NO: 1 can be used to determine whether a cell or sample contains *P. aeruginosa* MurE DNA or RNA. The effect of modulators that effect the transcription of the *murE* gene can be studied via the use of these probes. A preferred probe is a single stranded antisense probe having at least the full length of the coding sequence of MurE. It is also preferred to use probes that have less than the full length sequence, and contain sequences specific for *P. aeruginosa murE* DNA or RNA. The identification of a sequence(s) for use as a specific probe is well known in the art and involves choosing a sequence(s) that is unique to the target sequence, or is specific

thereto. It is preferred that polynucleotides that are probes have at least about 25 nucleotides, more preferably about 30 to 35 nucleotides. The longer probes are believed to be more specific for *P. aeruginosa murE* gene(s) and RNAs and can be used under more stringent hybridization conditions. Longer probes can be used but can be more difficult to prepare synthetically, or can result in lower yields from a synthesis. Examples of sequences that are useful as probes or primers for *P. aeruginosa murE*gene(s) are Primer A (sense) 5- TTTCATATGCCTATGAGCCTGAGCCAAC - 3' (SEQ ID NO:3) and Primer B (antisense) 5'- TTTGGATCCTCAAGCATGCGGCACCTC -3' (SEQ ID NO:4). These primers are nucleotides 1-22 (A) and the complement of nucleotides 1447-1464 (B) respectively, of SEQ ID NO:1. Restriction sites, underlined, for NdeI and BamHI are added to the 5' ends of the primers to allow cloning between the NdeI and BamHI sites of the expression vector pET-15b. However, one skilled in the art will recognize that these are only a few of the useful probe or primer sequences that can be derived from SEQ ID NO:1.

Polynucleotides having sequences that are unique or specific for *P. aeruginosa murE*can be used as primers in amplification reaction assays. These assays can be used in tissue typing as described herein. Additionally, amplification reactions employing primers derived from *P. aeruginosa murE* sequences can be used to obtain amplified *P. aeruginosa murE* DNA using the *murE* DNA of the cells as an initial template. The *murE* DNA so obtained can be a mutant or polymorphic form of *P. aeruginosa murE* that differs from SEQ ID NO:1 by one or more nucleotides of the MurE open reading frame or sequences flanking the ORF. The differences can be associated with a non-defective naturally occurring form or with a defective form of MurE. Thus, polynucleotides of this invention can be used in identification of various polymorphic *P. aeruginosa murE* genes or the detection of an organism having a *P. aeruginosa murE* gene. Many types of amplification reactions are known in the art and include, without limitation, Polymerase Chain Reaction, Reverse Transcriptase Polymerase Chain Reaction, Strand Displacement Amplification and Self-Sustained Sequence Reaction. Any of these or like reactions can be used with primers derived from SEQ ID NO:1.

Expression of MurE

A variety of expression vectors can be used to express recombinant MurE in host cells. Expression vectors are defined herein as nucleic acid sequences

that include regulatory sequences for the transcription of cloned DNA and the translation of their mRNAs in an appropriate host. Such vectors can be used to express a bacterial gene in a variety of hosts such as bacteria, bluegreen algae, plant cells, insect cells and animal cells. Specifically designed vectors allow the shuttling of genes between hosts such as bacteria-yeast or bacteria-animal cells. An appropriately constructed expression vector should contain: an origin of replication for autonomous replication in host cells, selectable markers, a limited number of useful restriction enzyme sites, a potential for high copy number, and regulatory sequences. A promoter is defined as a regulatory sequence that directs RNA polymerase to bind to DNA and initiate RNA synthesis. A strong promoter is one which causes mRNAs to be initiated at high frequency. Expression vectors can include, but are not limited to, cloning vectors, modified cloning vectors, specifically designed plasmids or viruses.

In particular, a variety of bacterial expression vectors can be used to express recombinant MurE in bacterial cells. Commercially available bacterial expression vectors which are suitable for recombinant MurE expression include, but are not limited to pQE (Qiagen), pET11a or pET15b (Novagen), lambda gt11 (Invitrogen), and pKK223-3 (Pharmacia).

Alternatively, one can express *murE* DNA in cell-free transcription-translation systems, or *murE* RNA in cell-free translation systems. Cell-free synthesis of MurE can be in batch or continuous formats known in the art.

One can also synthesize MurE chemically, although this method is not preferred.

A variety of host cells can be employed with expression vectors to synthesize MurE protein. These can include *E. coli*, *Bacillus*, and *Salmonella*. Insect and yeast cells can also be appropriate.

Following expression of MurE in a host cell, MurE polypeptides can be recovered. Several protein purification procedures are available and suitable for use. MurE protein and polypeptides can be purified from cell lysates and extracts, or from culture medium, by various combinations of, or individual application of methods including ultrafiltration, acid extraction, alcohol precipitation, salt fractionation, ionic exchange chromatography, phosphocellulose chromatography, lecithin chromatography, affinity (*e.g.*, antibody or His-Ni) chromatography, size exclusion chromatography, hydroxylapatite adsorption chromatography and chromatography based on hydrophobic or hydrophillic interactions. In some

instances, protein denaturation and refolding steps can be employed. High performance liquid chromatography (HPLC) and reversed phase HPLC can also be useful. Dialysis can be used to adjust the final buffer composition.

The MurE protein itself is useful in assays to identify compounds that modulate the activity of the protein -- including compounds that inhibit the activity of the protein. The MurE protein is also useful for the generation of antibodies against the protein, structural studies of the protein, and structure/function relationships of the protein.

Modulators and Inhibitors of MurE

The present invention is also directed to methods for screening for compounds which modulate or inhibit a MurE protein. Compounds which modulate or inhibit MurE can be DNA, RNA, peptides, proteins, or non-proteinaceous organic or inorganic compounds or other types of molecules. Compounds that modulate the expression of DNA or RNA encoding MurE or are inhibitors of the biological function of MurE can be detected by a variety of assays. The assay can be a simple "yes/no" assay to determine whether there is a change in expression or function. The assay can be made quantitative by comparing the expression or function of a test sample with the levels of expression or function in a standard sample, that is, a control. A compound that is a modulator can be detected by measuring the amount of the MurE produced in the presence of the compound. An compound that is an inhibitor can be detected by measuring the specific activity of the MurE protein in the presence and absence of the compound.

The proteins, DNA molecules, RNA molecules and antibodies lend themselves to the formulation of kits suitable for the detection and anaysis of MurE. Such a kit would comprise a compartmentalized carrier suitable to hold in close confinement at least one container. The carrier would further comprise reagents such as recombinant MurE or anti- MurE antibodies suitable for detecting MurE. The carrier can also contain a means for detection such as labeled antigen or enzyme substrates or the like.

Pharmaceutical Compositions

Pharmaceutically useful compositions comprising a modulator or inhibitor of MurE can be formulated according to known methods such as by the admixture of a pharmaceutically acceptable carrier. Examples of such carriers and

methods of formulation can be found in Remington's Pharmaceutical Sciences. To form a pharmaceutically acceptable composition suitable for effective administration, such compositions will contain an effective amount of the inhibitor.

Therapeutic, prophylactic or diagnostic compositions of the invention are administered to an individual in amounts sufficient to treat, prevent or diagnose disorders. The effective amount can vary according to a variety of factors such as the individual's condition, weight, sex and age. Other factors include the mode of administration. The appropriate amount can be determined by a skilled physician

The pharmaceutical compositions can be provided to the individual by a variety of routes such as subcutaneous, topical, oral and intramuscular.

The term "chemical derivative" describes a molecule that contains additional chemical moieties which are not normally a part of the base molecule. Such moieties can improve the solubility, half-life, absorption, etc. of the base molecule. Alternatively the moieties can attenuate undesirable side effects of the base molecule or decrease the toxicity of the base molecule. Examples of such moieties are described in a variety of texts, such as Remington's Pharmaceutical Sciences.

Compounds identified according to the methods disclosed herein can be used alone at appropriate dosages. Alternatively, co-administration or sequential administration of other agents can be desirable.

The present invention also provides a means to obtain suitable topical, oral, systemic and parenteral pharmaceutical formulations for use in the methods of treatment of the present invention. The compositions containing compounds identified according to this invention as the active ingredient can be administered in a wide variety of therapeutic dosage forms in conventional vehicles for administration. For example, the compounds can be administered in such oral dosage forms as tablets, capsules (each including timed release and sustained release formulations), pills, powders, granules, elixirs, tinctures, solutions, suspensions, syrups and emulsions, or by injection. Likewise, they can also be administered in intravenous (both bolus and infusion), intraperitoneal, subcutaneous, topical with or without occlusion, or intramuscular form, all using forms well known to those of ordinary skill in the pharmaceutical arts.

Advantageously, compounds of the present invention can be administered in a single daily dose, or the total daily dosage can be administered in divided doses of two, three or four times daily. Furthermore, compounds for the present invention can be administered in intranasal form via topical use of suitable

intranasal vehicles, or via transdermal routes, using those forms of transdermal skin patches well known to those of ordinary skill in that art. To be administered in the form of a transdermal delivery system, the dosage administration will, of course, be continuous rather than intermittent throughout the dosage regimen.

For combination treatment with more than one active agent, where the active agents are in separate dosage formulations, the active agents can be administered concurrently, or they each can be administered at separately staggered times.

The dosage regimen utilizing the compounds of the present invention is selected in accordance with a variety of factors including type, species, age, weight, sex and medical condition of the patient; the severity of the condition to be treated; the route of administration; the renal, hepatic and cardiovascular function of the patient; and the particular compound thereof employed. A physician or veterinarian of ordinary skill can readily determine and prescribe the effective amount of the drug required to prevent, counter or arrest the progress of the condition. Optimal precision in achieving concentrations of drug within the range that yields efficacy without toxicity requires a regimen based on the kinetics of the drug's availability to target sites. This involves a consideration of the distribution, equilibrium, and elimination of a drug.

The following examples are presented by the way of illustration and, because various other embodiments will be apparent to those in the art, the following is not to be construed as a limitation on the scope of the invention. For example, while particular preferred embodiments of the invention are presented herein, it is within the ability of persons of ordinary skill in the art to modify or substitute vectors, host cells, compositions, etc., or to modify or design protocols or assays, all of which may reach the same or equivalent performance or results as the embodiments shown herein.

EXAMPLE 1

<u>General Materials and Methods</u>

All reagents were purchased from SIGMA CHEMICAL CO., St. Louis, MO, unless otherwise indicated. UDP-N-acetylmuramyl-L-alanine was synthesized and purified by a method known in the art (Jin, H., Emanuele, J. J., Jr.,

Fairman, R., Robertson, J. G., Hail, M. E., Ho, H.-T., Falk, P. and Villafranca, J. J, 1996. Structural studies of *Escherichia coli* UDP-N-acetylmuramate: L-alanine ligase, Biochemistry 35: 14423-14431).

DNA manipulations reagents and techniques. Restriction endonucleases and T4 ligase were obtained from GIBCO-BRL. Agarose gel electrophoresis and plasmid DNA preparations were performed according to published procedures (Sambrook, J., E. F. Fritsch, and T. Maniatis, 1989, Molecular cloning: a L, Laboratory Manual, 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory). Recombinant plasmids containing *P. aeruginosa murE* were propagated in *E. coli* DH5a (GIBCO-BRL, Rockville, MD) prior to protein expression in *E. coli* BL21(DE3)/plysS (NOVAGEN, Madison, WI). SDS-PAGE was performed with precast gels (NOVAGEN). DNA sequences were determined using an automated ABI PRISM™ DNA sequencer (PERKIN-ELMER ABI, Foster City, CA).

EXAMPLE 2

Cloning of *Pseudomonas aeruginosa murE*

Genomic DNA from *P. aeruginosa* (strain MB4439) was prepared from 100 ml late stationary phase culture in Brain Heart Infusion broth (DIFCO, Detroit, MI). Cells were washed with 0.2 M sodium acetate, suspended in 10 ml of TEG (100 mM Tris, pH 7, containing 10 mM EDTA and 25% glucose) and lysed by incubation with 200 μg of N-acetylmuramidase (Sigma) for 1h at 37°C. Chromosomal DNA was purified from the cell lysate using a QIAGEN (Santa Clarita, CA) genomic DNA preparation kit and following the manufacturers protocol. Briefly, the cell lysate was treated with protease K at 50°C for 45 min, loaded onto an equilibrated QIAGEN genomic tip, entered into the resin by centrifugation at 3000 rpm for 2 min. Following washing the genomic tip, the genomic DNA was eluted in distilled water and kept at 4°C. Approximately 50 ng genomic DNA was used as a template in PCR reactions to clone *murE*.

Two oligonucleotide primers (GIBCO/BRL, Bethesda, MD) complementary to sequences at the 5' and the 3' ends of *P. aeruginosa murE* were used to clone this gene using KLENTAQ ADVANTAGE™ polymerase (CLONTECH, Palo Alto, CA). The primer nucleotide sequences were as follows:

5'- TTTCATATGCCTATGAGCCTGAGCCAAC -3' (SEQ ID NO:3) (a NdeI linker plus nucleotides 1-22 of SEQ ID NO: 1) and
5'- TTTGGATCCTCAAGCATGCGGCACCTC -3' (SEQ ID NO:4) (a BamH1 linker plus the complement of nucleotides 1447-1464 of SEQ ID NO: 1). A PCR product representing *P. aeruginosa murE*was verified by nucleotide sequence, digested with NdeI and BamHI, and cloned between the NdeI and BamHI sites of pET-15b, creating plasmid pPaeMurE. This plasmid was used for expression of the *murE* gene in *E. coli.*

The plasmid pPaeMurE has been deposited with the American Type Culture Collection on ______, under the terms of the Budapest Treaty for the Deposit of Microorganisms and has been designated as ATCC ________________. The deposited material is provided as a convenience and is not an indication that the deposited material is required to describe or practice the invention. The sequence of the polynucleotide of the deposit, and the encoded amino acid sequence, are incorporated herein by reference and are controlling in the event of a conflict with any description of the sequences provided in this specification or the associated drawings. A license may be required to make, use, sell or offer to sell the polynucleotide of the deposit or a protein of the amino acid sequence encoded by the polynucleotide. No such license is granted herein.

EXAMPLE 3

Sequence analysis of *Pseudomonas aeruginosa murE*

The nucleotide sequence of *murE*, determined in both orientations, and the deduced amino acid sequence of the MurE protein is depicted in FIG. 1. Sequence comparison using the BLAST (Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. (1990) "Basic local alignment search tool." J. Mol. Biol. 215:403-410) algorithm against the GenBank database showed that, to varying degrees, the cloned region is homologous (65% similar, 48% identical) to *murE* gene from *E. coli* (Tao, J.S, and E. E., Ishiguro, 1989. Nucleotide sequence of the murE gene of *Escherichia coli*. Can. J. Microbiol. 35:1051-1054).

EXAMPLE 4

Overexpression, purification and enzymatic activity of *Pseudomonas aeruginosa* MurE

murE was cloned into the expression vector pET-15b (Novagen) as described above to create plasmid pPaeMurE. The pET-15b vector incorporates the 6xHistidine-tag into the protein construct to allow rapid purification of MurE by affinity chromatography. The pET (Plasmids for Expression by T7 RNA polymerase) plasmids are derived from pBR322 and designed for protein over-production in *E. coli*. The vector pET-15b contains the ampicillin resistance gene, ColE1 origin of replication in addition to T7 phage promoter and terminator. The T7 promoter is recognized by the phage T7 RNA polymerase but not by the *E. coli* RNA polymerase. A host *E coli* strain such as BL21(DE3)pLysS is engineered to contain integrated copies of T7 RNA polymerase under the control of lacUV5 that is inducible by IPTG. Production of a recombinant protein in the *E. coli* strain BL21(DE3)pLysS occurs after expression of T7RNA polymerase is induced.

The pPaeMurE plasmid was introduced into the host strain BL21 DE3/pLysS (NOVAGEN) for expression of His-tagged MurE. Colonies were grown at 37°C in 100 ml of LB broth containing 100 mg/ml ampicillin and 32 μg/ml chloramphenicol. When cultures reached a cell density of A_{600}=0.5, cells were pelleted and then resuspended in M9ZB medium (NOVAGEN) containing 1 mM IPTG. Cells were induced for 3 h at 30°C, pelleted at 3000*g*, and frozen at -80°C.

Cultures containing either the recombinant plasmid pPaeMurE or the control plasmid vector, pET-15b were grown at 30°C and induced with IPTG. Cells transformed with pPaeMurE contained an inducible protein of approximately 54.7 kDa, corresponding to the expected size of *P. aeruginosa* MurE protein as shown by SDS-PAGE. There were no comparable detectable protein bands after induction of cells transformed with the control plasmid vector, pET-15b.

Purification of recombinant MurE enzyme.

The cell pellet from 100 ml of induced culture prepared as described above was resuspended in 10 ml BT buffer (50 mM bis-tris-propane, pH 8.0, containing 100 mM potassium chloride and 1% glycerol) at 4°C. Cells were lysed either by freeze-thaw or by French Press. After centrifugation, the supernatant was

mixed with 15 ml of freshly prepared TALON (CLONTECH) resin and incubated for 30 min at room temp. The resin was washed twice by centrifugation with 25 ml of BT buffer at room temperature. Finally, the resin was loaded into a column and washed with 20 ml of BT, pH 7.0, containing 5 mM imidazole. Protein was eluted with 20 ml of BT buffer pH 8.0, containing 100 mM imidazole. Fractions (0.5 ml) were collected and analyzed by SDS-Gel electrophoresis.

Aliquots from cell lysates, either uninduced or induced with IPTG, and column-purified polypeptides were analyzed by SDS-PAGE. (FIG. 2) Lane 1, Molecular weight markers; Lane2, IPTG-induced lysate of cells (BL21(DE3)/pLysS) containing the control vector pET-15b; Lane 3, uninduced cell lysate containing the control vector pET-15b; lane 4, column-purified MurE; Lane 5 IPTG-induced lysate of cells expressing MurE; Lane 6, uninduced lysate of cells containing *murE*.

This resulted in a partially purified preparation of *P. aeruginosa* MurE protein that could be used in activity assays. The protein may be purified further, if desired, using methods known in the art.

Assay for activity of MurE enzyme

The ATP-dependent MurE activity was assayed by monitoring the formation of product ADP using the pyruvate kinase and lactate dehydrogenase coupled enzyme assay. The reaction was monitored spectrophotometrically.

Typically, the assay contained 100 mM BIS-TRIS-propane, pH 8.0, 200 μM NADH, 1 mM ATP, 20 mM PEP, 5 mM $MgCl_2$, 1 mM DTT, 350 μM UDP-N-acetyl-muramyl-L-alanine-D-Glutamine, 1 mM *m*-Dap, 33 units/ml of pyruvate kinase and 1660 units/ml of lactate dehydrogenase in a final volume of 200 or 400 μl. The mixture was incubated at 25°C for 5 min and the reaction initiated by the addition of 1-10 μg of MurE. These conditions are one example of an assay useful for evaluating the activity of MurE. Other assays can be used, or amounts of buffers, substrate and enzyme can be changed, as desired, to alter the rate of production of ADP.

ADP formation was monitored by the decrease in absorbance at 340 nm as a function of time using a MOLECULAR DEVICES SPECTRAMAXPLUS^{TM} microtiterplate spectrophotomer (for 200 μl assays) or a HEWLETT-PACKARD HP8452A spectrophotometer equipped with a circulating water bath (for 400 μl assays). Rates were calculated from the linear portions of the

progress curves using the extinction coefficient for NADH, $e = 6220\ cm^{-1}\ M^{-1}$. One unit of MurE activity is equal to 1 μmol of ADP formed per min at 25°C.

Table 1
Specific activities of recombinant MurE
from *E. coli* and *P. aeruginosa*

Mur Ligase	*P. aeruginosa* µmol x min^{-1} x mg^{-1}	*E. coli* µmol x min^{-1} x mg^{-1}
MurE	3.31	0.68

EXAMPLE 5

Screening for inhibitors of MurE

One assay for the measurement of the activity of MurE is provided in Example 4. That assay, and other assays for MurE activity can be adapted for screening assays to detect inhibitors of MurE. For example, for inhibition assays, inhibitors in DMSO are added at the desired concentration to the assay mixture. In a separate, control reaction, only DMSO is added to the assay mixture. The reactions are initiated by the addition of enzyme (MurE). Rates are calculated as described above. Relative activities are calculated from the equation 1:

relative activity = rate with inhibitor/rate without inhibitor. (1)

Inhibition constant (IC_{50}) values are determined from a range of inhibitor concentrations and calculated from equation 2.

$$\text{relative activity} = 1/(1 + [I]/IC_{50}) \qquad (2)$$

One can use computer software to assist in the analysis, *e.g.*, SIGMA PLOT™ (JANDEL SCIENTIFIC, San Rafeal, CA).

We prefer inhibitors of MurE that result in relative activities of the MurE enzyme of at least less than 75%, more preferably, 25-50% or 10-25%. We most prefer inhibitors resulting in relative activities of less than 20%, particularly less than 10% of the activity of MurE in the absence of the inhibitor.

We also prefer inhibitors that effectively lower the relative activity of MurE when the inhibitor is present at a very low concentration.

EXAMPLE 6

Therapy using inhibitors of MurE

A patient presenting with an indication of infection with a microorganism susceptible to inhibitors of MurE, *e.g.*, gram positive and negative bacteria, including *P. aeruginosa*, can be treated by administration of inhibitors of MurE. Physicians skilled in the art are familiar with administering therapeutically effective amounts of inhibitors or modulators of microbial enzymes. Such skilled persons can readily determine an appropriate dosing scheme to achieve a desired therapeutic effect.

Therapy can also be prophylactic. For example, a patient at risk for developing a bacterial infection, including infection with *P. aeruginosa*, can be treated by administration of inhibitors of MurE. Physicians skilled in the art are familiar with administering therapeutically effective amounts of inhibitors or modulators of microbial enzymes. Such skilled persons can readily determine an appropriate dosing scheme to achieve a desired therapeutic effect.

WHAT IS CLAIMED:

1. A purified and isolated polynucleotide selected from the group consisting of:

(a) a polynucleotide encoding a polypeptide having an amino acid sequence of SEQ ID NO: 2.

(b) a polynucleotide which is complementary to the polynucleotide of (a),

(c) a polynucleotide representing a naturally occurring mutant or polymorphic form of (a), and

(d) a polynucleotide that hybridizes with a polynucleotide of (a), (b), or (c) under stringent conditions, and

(e) a polynucleotide comprising at least 25 nucleotides of the polynucleotide of (a), (b) or (c), said 25 nucleotides being specific for *murE* gene of *Pseudomonas aeruginosa*.

2. The polynucleotide of claim 1 wherein the polynucleotide comprises nucleotides selected from the group consisting of natural, non-natural and modified nucleotides.

3. The polynucleotide of claim 1 wherein the internucleotide linkages are selected from the group consisting of natural and non-natural linkages.

4. The polynucleotide of claim 1 comprising the nucleotide sequence of SEQ ID NO:1.

5. A polynucleotide that is an expression vector comprising a polynucleotide of claim 1.

6. A host cell comprising the expression vector of claim 5.

7. A process for expressing a MurE protein of *Pseudomonas aeruginosa* in a recombinant host cell, comprising:

(a) transforming a suitable host cell with an expression vector of claim 5; and,

(b) culturing the host cell of step (a) in conditions under which allow expression of said the MurE protein from said expression vector.

8. A purified and isolated polypeptide having an amino acid sequence selected from the group consisting of

(a) a polypeptide having an amino acid sequence of SEQ ID NO:2,

(b) a polypeptide that is a naturally occurring mutant or polymorphic form of (a).

9. A method of determining whether a candidate compound is an inhibitor of a *Pseudomonas aeruginosa* MurE polypeptide comprising:

(a) providing at least one host cell harboring an expression vector that includes a polynucleotide selected from the group consisting of:

(i) a polynucleotide encoding a polypeptide having an amino acid sequence of SEQ ID NO: 2.

(ii) a polynucleotide which is complementary to the polynucleotide of (i),

(iii) a polynucleotide representing a naturally occurring mutant or polymorphic form of (i), and

(b) contacting at least one of said cells with the candidate to permit the interaction of the candidate with the MurE polypeptide, and

(c) determining whether the candidate is an inhibitor of the MurE polypeptide by ascertaining the relative activity of the polypeptide in the presence of the candidate.

10. The method of claim 9 wherein the polynucleotide has the nucleotide sequence of SEQ ID NO:1.

11. The method of claim 9 wherein in step (c) the relative activity is determined by comparing a measurement of MurE polypeptide activity of at least one cell before step (b) to a measurement of MurE polypeptide activity of at least one cell after step (b).

12. A compound that is an inhibitor of a polypeptide having an amino acid sequence selected from the group consisting of

(a) a polypeptide having an amino acid sequence of SEQ ID NO:2,

(b) a polypeptide that is a naturally occurring mutant or polymorphic form of (a).

13. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and an inhibitor of a polypeptide having an amino acid sequence selected from the group consisting of

(a) a polypeptide having an amino acid sequence of SEQ ID NO:2,

(b) a polypeptide that is a naturally occurring mutant or polymorphic form of (a).

14. A method of treatment of a patient in need of prophylactic or therapeutic treatment for a bacterial infection comprising administering to the patient an effective amount of an inhibitor of a polypeptide having an amino acid sequence selected from the group consisting of

(a) a polypeptide having an amino acid sequence of SEQ ID NO:2,

(b) a polypeptide representing a naturally occurring mutant or polymorphic form of (a).

15. A method of determining whether a candidate compound is an inhibitor of a *Pseudomonas aeruginosa* MurE polypeptide comprising:

(a) providing a sample that includes a MurE polypeptide selected from the group consisting of:

(i) a polypeptide having an amino acid sequence of SEQ ID NO: 2.

(ii) a polypeptide that is a functional derivative of the polypeptide of (i),

(iii) a polypeptide representing a naturally occurring mutant or polymorphic form of (i), and

(b) contacting said sample with the candidate to permit the interaction of the candidate with the MurE polypeptide, and

(c) determining whether the candidate is an inhibitor of the MurE polypeptide by ascertaining the relative activity of the MurE polypeptide in the presence of the candidate.

16. The method of claim 15 wherein the polypeptide has the amino acid sequence of SEQ ID NO:2.

17. The method of claim 15 wherein in step (c) the relative activity is determined by comparing a measurement of MurE polypeptide activity of the sample before step (b) to a measurement of MurE polypeptide activity of the sample after step (b)

FIG. 1A

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  1 ATGCCTATGAGCCTGAGCCAACTGTTTCCCCAGGCCGAGCGCGATCTGCTGATCCGCGAG   60
    TACGGATACTCGGACTCGGTTGACAAAGGGGTCCGGCTCGCGCTAGACGACTAGGCGCTC
    MetProMetSerLeuSerGlnLeuPheProGlnAlaGluArgAspLeuLeuIleArgGlu

 61 CTGACCCTGGATAGCCACGGCGTTCGTCCGGTCGACCTGTTCCTGACGGTTCCGGGCGGG  120
    GACTGGGACCTATCGGTGCCGCAAGCAGGCCAGCTGGACAAGGACTGCCAAGGCCCGCCC
    LeuThrLeuAspSerHisGlyValArgProValAspLeuPheLeuThrValProGlyGly

121 CACCAGGATGGTCGTGCGCACATCGCCGATGCCCTGACCAAGGGCGCGACTGCCGTGGCT  180
    GTGGTCCTACCAGCACGCGTGTAGCGGCTACGGGACTGGTTCCCGCGCTGACGGCACCGA
    HisGlnAspGlyArgAlaHisIleAlaAspAlaLeuThrLysGlyAlaThrAlaValAla

181 TACGAGGCGGAAGGCGCCGGAGAGTTGCCGCCCAGCGATGCGCCGCTGATCGCGGTGAAG  240
    ATGCTCCGCCTTCCGCGGCCTCTCAACGGCGGGTCGCTACGCGGCGACTAGCGCCACTTC
    TyrGluAlaGluGlyAlaGlyGluLeuProProSerAspAlaProLeuIleAlaValLys

241 GGGCTGGCCGCGCAACTGTCGGCGGTCGCCGGGCGTTTCTACGGCGAGCCGAGCCGCGGG  300
    CCCGACCGGCGCGTTGACAGCCGCCAGCGGCCCGCAAAGATGCCGCTCGGCTCGGCGCCC
    GlyLeuAlaAlaGlnLeuSerAlaValAlaGlyArgPheTyrGlyGluProSerArgGly

301 CTGGACCTGATCGGCGTCACCGGCACCAACGGCAAGACCAGCGTCAGCCAACTGGTGGCC  360
    GACCTGGACTAGCCGCAGTGGCCGTGGTTGCCGTTCTGGTCGCAGTCGGTTGACCACCGG
    LeuAspLeuIleGlyValThrGlyThrAsnGlyLysThrSerValSerGlnLeuValAla

361 CAGGCCCTGGATCTGCTCGGCGAGCGCTGCGGCATCGTCGGCACCCTCGGCACCGGTTTC  420
    GTCCGGGACCTAGACGAGCCGCTCGCGACGCCGTAGCAGCCGTGGGAGCCGTGGCCAAAG
    GlnAlaLeuAspLeuLeuGlyGluArgCysGlyIleValGlyThrLeuGlyThrGlyPhe

421 TACGGCGCCCTGGAGAGCGGCCGGCACACCACGCCGGACCCGCTCGCGGTGCAGGCCACG  480
    ATGCCGCGGGACCTCTCGCCGGCCGTGTGGTGCGGCCTGGGCGAGCGCCACGTCCGGTGC
    TyrGlyAlaLeuGluSerGlyArgHisThrThrProAspProLeuAlaValGlnAlaThr

481 CTGGCCACGCTGAAGCAGGCCGGCGCCCGCGCGGTAGCGATGGAAGTGTCTTCCCACGGC  540
    GACCGGTGCGACTTCGTCCGGCCGCGGGCGCGCCATCGCTACCTTCACAGAAGGGTGCCG
    LeuAlaThrLeuLysGlnAlaGlyAlaArgAlaValAlaMetGluValSerSerHisGly

541 CTCGACCAGGGCCGCGTGGCGGCGCTCGGCTTCGATATCGCGGTGTTCACCAATCTGTCC  600
    GAGCTGGTCCCGGCGCACCGCCGCGAGCCGAAGCTATAGCGCCACAAGTGGTTAGACAGG
    LeuAspGlnGlyArgValAlaAlaLeuGlyPheAspIleAlaValPheThrAsnLeuSer
```

FIG. 1B

```
601  CGCGACCACCTCGACTATCACGGTTCGATGGAAGCCTATGCCGCCGCCAAGGCCAAGCTG  660
     GCGCTGGTGGAGCTGATAGTGCCAAGCTACCTTCGGATACGGCGGCGGTTCCGGTTCGAC
     ArgAspHisLeuAspTyrHisGlySerMetGluAlaTyrAlaAlaAlaLysAlaLysLeu

661  TTCGCCTGGCCGGACCTGCGCTGCCGGGTGATCAACCTGGACGACGATTTCGGCCGTCGA  720
     AAGCGGACCGGCCTGGACGCGACGGCCCACTAGTTGGACCTGCTGCTAAAGCCGGCAGCT
     PheAlaTrpProAspLeuArgCysArgValIleAsnLeuAspAspAspPheGlyArgArg

721  CTGGCCGGCGAGGAGCAGGACTCGGAGCTGATCACCTACAGCCTCACCGACAGCTCGGCG  780
     GACCGGCCGCTCCTCGTCCTGAGCCTCGACTAGTGGATGTCGGAGTGGCTGTCGAGCCGC
     LeuAlaGlyGluGluGlnAspSerGluLeuIleThrTyrSerLeuThrAspSerSerAla

781  TTCCTCTATTGCCGCGAAGCGCGCTTCGGCGACGCCGGCATCGAGGCGGCGCTGGTCACT  840
     AAGGAGATAACGGCGCTTCGCGCGAAGCCGCTGCGGCCGTAGCTCCGCCGCGACCAGTGA
     PheLeuTyrCysArgGluAlaArgPheGlyAspAlaGlyIleGluAlaAlaLeuValThr

841  CCGCACGGCGAGGGCCTGCTGCGCAGCCCGTTGCTCGGCCGCTTCAACCTGAGCAACCTG  900
     GGCGTGCCGCTCCCGGACGACGCGTCGGGCAACGAGCCGGCGAAGTTGGACTCGTTGGAC
     ProHisGlyGluGlyLeuLeuArgSerProLeuLeuGlyArgPheAsnLeuSerAsnLeu

901  CTGGCGGCGGTCGGTGCGTTGCTTGGCCTGGGTTATCCCCTGGGCGATATCCTCCGCACT  960
     GACCGCCGCCAGCCACGCAACGAACCGGACCCAATAGGGGACCCGCTATAGGAGGCGTGA
     LeuAlaAlaValGlyAlaLeuLeuGlyLeuGlyTyrProLeuGlyAspIleLeuArgThr

961  TTGCCGCAACTGCAGGGGCCGGTCGGCCGCATGCAGCGCCTGGGAGGCGGCGGCAAGCCG  1020
     AACGGCGTTGACGTCCCCGGCCAGCCGGCGTACGTCGCGGACCCTCCGCCGCCGTTCGGC
     LeuProGlnLeuGlnGlyProValGlyArgMetGlnArgLeuGlyGlyGlyGlyLysPro

1021 CTGGTGGTGGTGGACTACGCGCATACTCCCGACGCCCTGGAAAAAGTCCTGGAGGCCCTG  1080
     GACCACCACCACCTGATGCGCGTATGAGGGCTGCGGGACCTTTTTCAGGACCTCCGGGAC
     LeuValValValAspTyrAlaHisThrProAspAlaLeuGluLysValLeuGluAlaLeu

1081 CGTCCGCACGCGGCCGCGCGCCTGCTGTGCCTGTTCGGCTGCGGTGGCGATCGCGATGCC  1140
     GCAGGCGTGCGCCGGCGCGCGGACGACACGGACAAGCCGACGCCACCGCTAGCGCTACGG
     ArgProHisAlaAlaAlaArgLeuLeuCysLeuPheGlyCysGlyGlyAspArgAspAla

1141 GGCAAGCGTCCGCTGATGGCTGCGATCGCCGAACGCCTGGCGGATGAGGTGCTGGTCACC  1200
     CCGTTCGCAGGCGACTACCGACGCTAGCGGCTTGCGGACCGCCTACTCCACGACCAGTGG
     GlyLysArgProLeuMetAlaAlaIleAlaGluArgLeuAlaAspGluValLeuValThr
```

FIG. 1C

```
1201 GACGACAACCCGCGCACCGAGGCCAGTGCGGCGATCATCGCCGATATCCGCAAAGGCTTC 1260
     CTGCTGTTGGGCGCGTGGCTCCGGTCACGCCGCTAGTAGCGGCTATAGGCGTTTCCGAAG
     AspAspAsnProArgThrGluAlaSerAlaAlaIleIleAlaAspIleArgLysGlyPhe

1261 GCTGCCGCTGACAAGGTTACCTTCCTGCCGTCGCGCGGTGAGGCGATCGCCCATCTGATC 1320
     CGACGGCGACTGTTCCAATGGAAGGACGGCAGCGCGCCACTCCGCTAGCGGGTAGACTAG
     AlaAlaAlaAspLysValThrPheLeuProSerArgGlyGluAlaIleAlaHisLeuIle

1321 GCTTCCGCTGCGGTGGATGACGTGGTGCTCCTGGCCGGCAAGGGTCACGAGGATTATCAG 1380
     CGAAGGCGACGCCACCTACTGCACCACGAGGACCGGCCGTTCCCAGTGCTCCTAATAGTC
     AlaSerAlaAlaValAspAspValValLeuLeuAlaGlyLysGlyHisGluAspTyrGln

1381 GAGATCGACGGCGTACGCCATCCGTTCTCCGACATCGAGCAGGCCGAGCGCGCCCTGGCC 1440
     CTCTAGCTGCCGCATGCGGTAGGCAAGAGGCTGTAGCTCGTCCGGCTCGCGCGGGACCGG
     GluIleAspGlyValArgHisProPheSerAspIleGluGlnAlaGluArgAlaLeuAla

1441 GCCTGGGAGGTGCCGCATGCTTGAGCCTCTTCGCCTCAGCCAGTTGACGGTCGCGCTGG 1500
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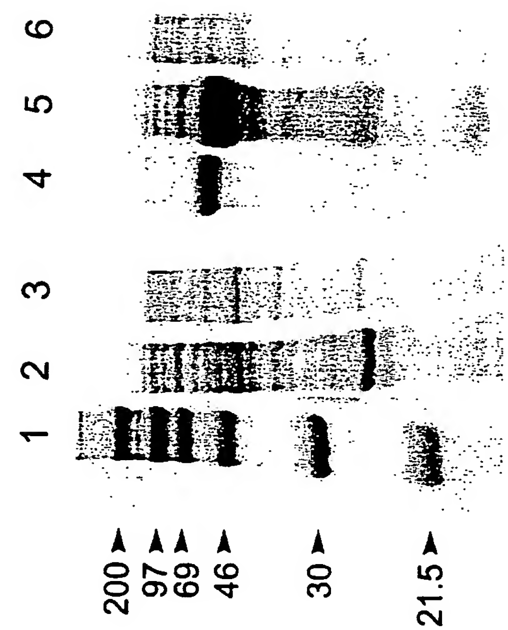
FIG. 2



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Pro Ser Arg Gly Leu Asp Leu Ile Gly Val Thr Gly Thr Asn Gly Lys
            100                 105                 110
Thr Ser Val Ser Gln Leu Val Ala Gln Ala Leu Asp Leu Leu Gly Glu
        115                 120                 125
Arg Cys Gly Ile Val Gly Thr Leu Gly Thr Gly Phe Tyr Gly Ala Leu
    130                 135                 140
Glu Ser Gly Arg His Thr Thr Pro Asp Pro Leu Ala Val Gln Ala Thr
145                 150                 155                 160
Leu Ala Thr Leu Lys Gln Ala Gly Ala Arg Ala Val Ala Met Glu Val
                165                 170                 175
Ser Ser His Gly Leu Asp Gln Gly Arg Val Ala Ala Leu Gly Phe Asp
            180                 185                 190
Ile Ala Val Phe Thr Asn Leu Ser Arg Asp His Leu Asp Tyr His Gly
        195                 200                 205
Ser Met Glu Ala Tyr Ala Ala Ala Lys Ala Lys Leu Phe Ala Trp Pro
    210                 215                 220
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225                 230                 235                 240
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                245                 250                 255
Asp Ser Ser Ala Phe Leu Tyr Cys Arg Glu Ala Arg Phe Gly Asp Ala
            260                 265                 270
Gly Ile Glu Ala Ala Leu Val Thr Pro His Gly Glu Gly Leu Leu Arg
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                325                 330                 335
Gly Gly Lys Pro Leu Val Val Val Asp Tyr Ala His Thr Pro Asp Ala
            340                 345                 350
Leu Glu Lys Val Leu Glu Ala Leu Arg Pro His Ala Ala Ala Arg Leu
        355                 360                 365
Leu Cys Leu Phe Gly Cys Gly Gly Asp Arg Asp Ala Gly Lys Arg Pro
    370                 375                 380
Leu Met Ala Ala Ile Ala Glu Arg Leu Ala Asp Glu Val Leu Val Thr
385                 390                 395                 400
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            420                 425                 430
Gly Glu Ala Ile Ala His Leu Ile Ala Ser Ala Ala Val Asp Asp Val
        435                 440                 445
Val Leu Leu Ala Gly Lys Gly His Glu Asp Tyr Gln Glu Ile Asp Gly
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INTERNATIONAL SEARCH REPORT

International applicati n No.
PCT/US00/24743

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C07H 21/04; C12P 21/06; C12N 15/09, 15/00.
US CL :536/23.1, 23.9; 435/71.1, 69.1, 69.3, 320.1.
According to Internati nal Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.1, 23.9; 435/71.1, 69.1, 69.3, 320.1.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, EMBASE, DIALOG, WEST, Sequence Databeses.
SEQ ID NO: 1, MurE, aeruginosa, inventors' names.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LIAO et al. Cloning and characterization of the Pseudomonas aeruginosa pbpB gene encoding penicillin-binding protein 3. Antimicrob. Agents Chemother. August 1995, Vol. 39, No. 8, pages 1871-1874, see entire document.	1-6

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
04 DECEMBER 2000	02 JAN 2001

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Dorthea Lawrence For S. DEVI, Ph.D.
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/24743

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-6

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/24743

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claims 1-6, drawn to an isolated polynucleotide encoding a polypeptide having SEQ ID NO: 2, or that is complementary to the polynucleotide or that hybridizes with the polynucleotide, a cDNA, an expression vector and a host cell comprising the same.
Group II, claim 7, drawn to a process for expressing a MurE protein of *Pseudomonas aeruginosa* using the expression vector comprising the polynucleotide.
Group III, claims 8 and 15-17, drawn to an isolated polypeptide comprising an amino acid sequence of SEQ ID NO: 2 and a method of using the polypeptide.
Group IV, claims 9-11, drawn to a method of determining whether a compound is an inhibitor of *Ps. aeruginosa* MurE polypeptide by providing the expression vector.
Group V, claims 12-14, drawn to a compound that is an inhibitor of polypeptide having an amino acid sequence of SEQ ID NO: 2 and a method of treatment by administering an inhibitor of a polypeptide having an amino acid sequence of SEQ ID NO: 2.

The special technical feature of Invention I is an isolated polynucleotide that is complementary to the polynucleotide or that hybridizes with the polynucleotide of SEQ ID NO: 1. Invention II is drawn to a process for expressing a MurE protein of *Ps. aeruginosa* using the product of invention I. Although the first product of the invention and method of using the product is a permitted combination under PCT Rule 13.2, in the instant case, the special technical feature is already disclosed in the art, for instance, by Liao *et al. Antimicrob. Agents Chemother*. 39 (8): 1871-1874, 1995 and therefore is not a unifying feature. Invention III is drawn to a second product, polypeptide, and a method of using the product. Invention IV is drawn to a second method of use of the expression vector comprising the polynucleotide. G ›up V is drawn to a third product, a compound that inhibits the polypeptide, and a method of using the product. Clearly, the special technical feature is not a unifying feature.

PATENT COOPERATI TREATY

4

PCT

REC'D 09 NOV 2001
WIPO PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 20195-PCT	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/US00/24743	International filing date *(day/month/year)* 11 SEPTEMBER 2000	Priority date *(day/month/year)* 15 SEPTEMBER 1999
International Patent Classification (IPC) or national classification and IPC Please See Supplemental Sheet.		
Applicant MERCK & CO., INC.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.
2. This REPORT consists of a total of 5 sheets.

   ☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 0 sheets.

3. This report contains indications relating to the following items:

   I ☒ Basis of the report
   II ☐ Priority
   III ☒ Non-establishment of report with regard to novelty, inventive step or industrial applicability
   IV ☐ Lack of unity of invention
   V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   VI ☐ Certain documents cited
   VII ☐ Certain defects in the international application
   VIII ☐ Certain observations on the international application

Date of submission of the demand 29 MARCH 2001	Date of completion of this report 21 SEPTEMBER 2001
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Dorothea Lawrence for S. DEVI, Ph.D. Telephone No. (703) 308-0196

Form PCT/IPEA/409 (cover sheet) (July 1998) ★

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US00/24743

I. Basis of the report

1. With regard to the elements of the international application:*

[X] the international application as originally filed

[X] the description:

pages 1-26 , as originally filed

pages NONE , filed with the demand

pages NONE , filed with the letter of ____

[X] the claims:

pages 27-30 , as originally filed

pages NONE , as amended (together with any statement) under Article 19

pages NONE , filed with the demand

pages NONE , filed with the letter of ____

[X] the drawings:

pages 1-4 , as originally filed

pages NONE , filed with the demand

pages NONE , filed with the letter of ____

[X] the sequence listing part of the description:

pages 1-3 , as originally filed

pages NONE , filed with the demand

pages NONE , filed with the letter of ____

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language ____ which is:

[ ] the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

[ ] the language of publication of the international application (under Rule 48.3(b)).

[ ] the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

[X] contained in the international application in printed form.

[X] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readable form.

[ ] The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] The statement that the information recorded in computer readable form is identical to the writen sequence listing has been furnished.

4. [X] The amendments have resulted in the cancellation of:

[X] the description, pages NONE

[X] the claims, Nos. NONE

[X] the drawings, sheets/~~fig~~ NONE

5. [ ] This report has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

* *Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

***Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.*

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US00/24743

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non obvious), or to be industrially applicable have not been and will not be examined in respect of:

☐ the entire international application.

☒ claims Nos. 7-17

because:

☐ the said international application, or the said claim Nos. _ relate to the following subject matter which does not require international preliminary examination *(specify)*.

☐ the description, claims or drawings *(indicate particular elements below)* or said claims Nos. _ are so unclear that no meaningful opinion could be formed *(specify)*.

☐ the claims, or said claims Nos. _ are so inadequately supported by the description that no meaningful opinion could be formed.

☒ no international search report has been established for said claims Nos. 7-17.

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

☐ the written form has not been furnished or does not comply with the standard.

☐ the computer readable form has not been furnished or does not comply with the standard.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US00/24743

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. statement

Novelty (N)	Claims	4	YES
	Claims	1-3, 5 and 6	NO
Inventive Step (IS)	Claims	4	YES
	Claims	1-3, 5 and 6	NO
Industrial Applicability (IA)	Claims	1-6	YES
	Claims	NONE	NO

2. citations and explanations (Rule 70.7)

Claims 1-3, 5 and 6 lack novelty under PCT Article 33(2) as being anticipated by Liao et al.

Liao et al. teach a polynucleotide comprising at least 25 nucleotides of the instantly claimed SEQ ID NO: 1, an expression vector and a host cell comprising the same. Liao's polynucleotide shows high degree of homology to the instantly claimed SEQ ID NO: 1. That Liao's polynucleotide comprises natural linkages and that it is complementary to SEQ ID NO: 1 is inherent from the teachings of Liao et al.

Claim 4 meets the criteria set out in PCT Article 33(2)-(4), because the prior art does not teach or fairly suggest a polynucleotide comprising the nucleotide sequence of SED ID NO: 1.

-------------------- NEW CITATIONS --------------------
NONE

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

Sheet 10

CLASSIFICATION:

The International Patent Classification (IPC) and/or the National classification are as listed below:
IPC(7): C07H 21/04; C12P 21/06; C12N 15/09, 15/00. and US Cl.: 536/23.1, 23.9; 435/71.1, 69.1, 69.3, 320.1.

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Aug. 1995, p. 1871–1874
0066-4804/95/$04.00+0

Vol. 39, No. 8

Cloning and Characterization of the *Pseudomonas aeruginosa* *pbpB* Gene Encoding Penicillin-Binding Protein 3

XIAOWEN LIAO AND ROBERT E. W. HANCOCK*

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Clones containing the *pbpB* gene which encodes penicillin-binding protein (PBP) 3 of *Pseudomonas aeruginosa* were detected by hybridization by PCR amplification with primers based on the conserved sequences of high-molecular-weight PBPs. The translated amino acid sequence demonstrated 45% identity and had a total of 66% conserved amino acids relative to the *Escherichia coli* PBP3. The *pbpB* gene was located upstream of a gene homologous to the *E. coli murE* gene, which encodes uridine diphosphate-*N*-acetyl muramic acid-tripeptide synthetase. The overexpressed *pbpB* gene product reacted with ^{3}H-penicillin G and had an apparent molecular weight of 60,000.

Pseudomonas aeruginosa is an opportunistic pathogen that is intrinsically resistant to a wide range of antibiotics. Infections by this bacterium are often treated with β-lactam antibiotics. β-Lactams exert their effects by acting as the substrate analogs of the peptidoglycan biosynthetic enzymes transpeptidase and D-alanine carboxypeptidase (21), which are located within the cytoplasmic membrane and are commonly named penicillin-binding proteins (PBPs) because of their abilities to covalently bind radiolabelled penicillin (16). It has been found that there are six to seven PBPs in *P. aeruginosa* (14). However, PBPs of *P. aeruginosa* are not as well studied as those in *Escherichia coli* (17, 18) and their genes have not been characterized. Maejima et al. (10) and Watanabe et al. (23) reported that *P. aeruginosa* PBP3 was the primary target for the expanded-spectrum and "fourth generation" cephalosporins. It was observed by Godfrey et al. (5) that some β-lactam-resistant clinical isolates of *P. aeruginosa* from cystic fibrosis patients had apparently lost PBP3 and/or PBP6. Therefore, it seems highly likely that *P. aeruginosa* PBP3 plays an important role in susceptibility to β-lactam antibiotics. To further characterize this protein, in this study we have cloned and sequenced the *pbpB* gene encoding PBP3.

P. aeruginosa PAO1 strain H103 (6) was used for cloning. All DNA techniques were performed essentially as described previously (2, 11), except for isolation of chromosomal DNA, for which hexadecyltrimethyl ammonium bromide was used (1). DNA fragments were isolated by using the Geneclean kit (BIO 101 Inc., La Jolla, Calif.). DNA probes were labelled with digoxigenin-dUTP (Boehringer Mannheim) by random primer labelling. Southern hybridization and colony hybridization were performed by using the Genius system (Boehringer Mannheim). LB broth (0.8% Bacto-tryptone, 0.5% yeast extract) with 0.5 or 0.05% NaCl was used for *E. coli* and *P. aeruginosa* growth, respectively; 2% Bacto-agar was added when solid medium was required. Antibiotics used in selection media included ampicillin at 75 μg/ml, chloramphenicol at 30 μg/ml, and kanamycin at 50 μg/ml.

Although the amino acid sequences of PBPs are only moderately conserved, the sequences around the active-site motif S*XXK (where S* is the binding residue) and the KTG motif are highly homologous among the high-molecular-weight PBPs of *E. coli* and *Neisseria gonorrhoeae* PBP2. Primers for PCR were designed according to the sequences at and surrounding the conserved motifs S*XXK and KTG of *E. coli* PBP1A, -1B, -2, and -3 and *N. gonorrhoeae* PBP2 (18), adjusted to the codon usage of *P. aeruginosa* (24). The sequence of the degenerate upstream primer based on the S*XXK motif was 5′-TTTG AATTCGG(C)CA(T)C(G)C(G)G(AC)C(AT)G(C)G(A)C(T) G(C)AAGCC-3′, which corresponded to the amino acid sequence G(A)ST(ANL)V(IAM)KP. The sequence of the degenerate downstream primer based on the KTG motif was 5′-AAAGAATTCG(CT)T(C)T(G)C(G)GT(C)C(G)GTGCC G(C)G(C)T(A)CTT-3′, which corresponded to the amino acid sequence KT(S)GTT(A)N(QRK). Both of these primers were synthesized with nine extra nucleotides at the 5′ end containing an *Eco*RI recognition site, which ensured that the PCR products could be subsequently digested by *Eco*RI and then cloned into the vector pTZ18U. The PCR was performed in the presence of 5% formamide, 10% glycerol, and 15 mM Mg^{2+}, under conditions whereby the first 5 cycles involved temperature cycles of 94°C for 15 s, 37°C for 30 s, and 72°C for 90 s whereas for the remaining 25 cycles the primer-annealing temperature was raised from 37 to 55°C. A mixture of PCR products ranging from 200 to 750 bp was obtained. All of these products were gel purified, digested with *Eco*RI, and cloned into a vector, pTZ18U. One of these PCR products, 580 bp in length, was found to translate to a sequence with 71 and 72% conservation of amino acids compared with *E. coli* PBP3 and *N. gonorrheae* PBP2, respectively. None of the other PCR products demonstrated any homology to PBP1A, -1B, or -2 of *E. coli*. With the 580-bp PCR product as a probe, Southern blots of the *P. aeruginosa* PAO1 chromosome DNA, which had been digested with various restriction enzymes individually or in combination, permitted the creation of a restriction enzyme map (Fig. 1A). In our hands, the entire gene could not be cloned as a single fragment. Therefore, restriction fragments containing three different portions of the PAO1 chromosomal DNA corresponding to the 1.4-kb *Sph*I, 1.8-kb *Pst*I, and 4.4-kb *Sma*I-*Xho*I fragments were cloned into a vector, pTZ19U, respectively. The positive clones pSPH1, pPST18, and pXSm16 were detected by colony hybridization (Fig. 1B). None of these clones contained the entire gene. Therefore, a 1-kb *Sma*I fragment from plasmid pSPH1 was

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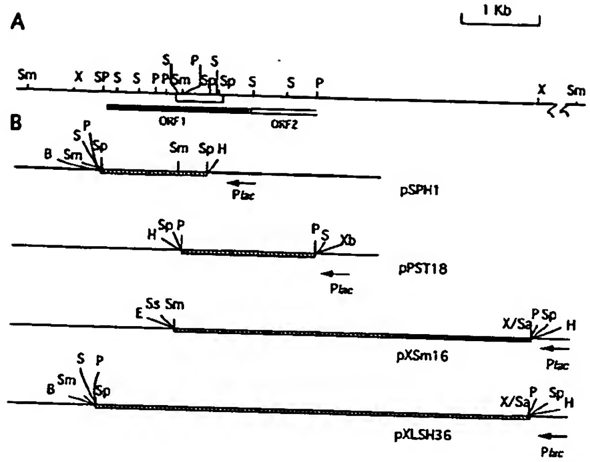

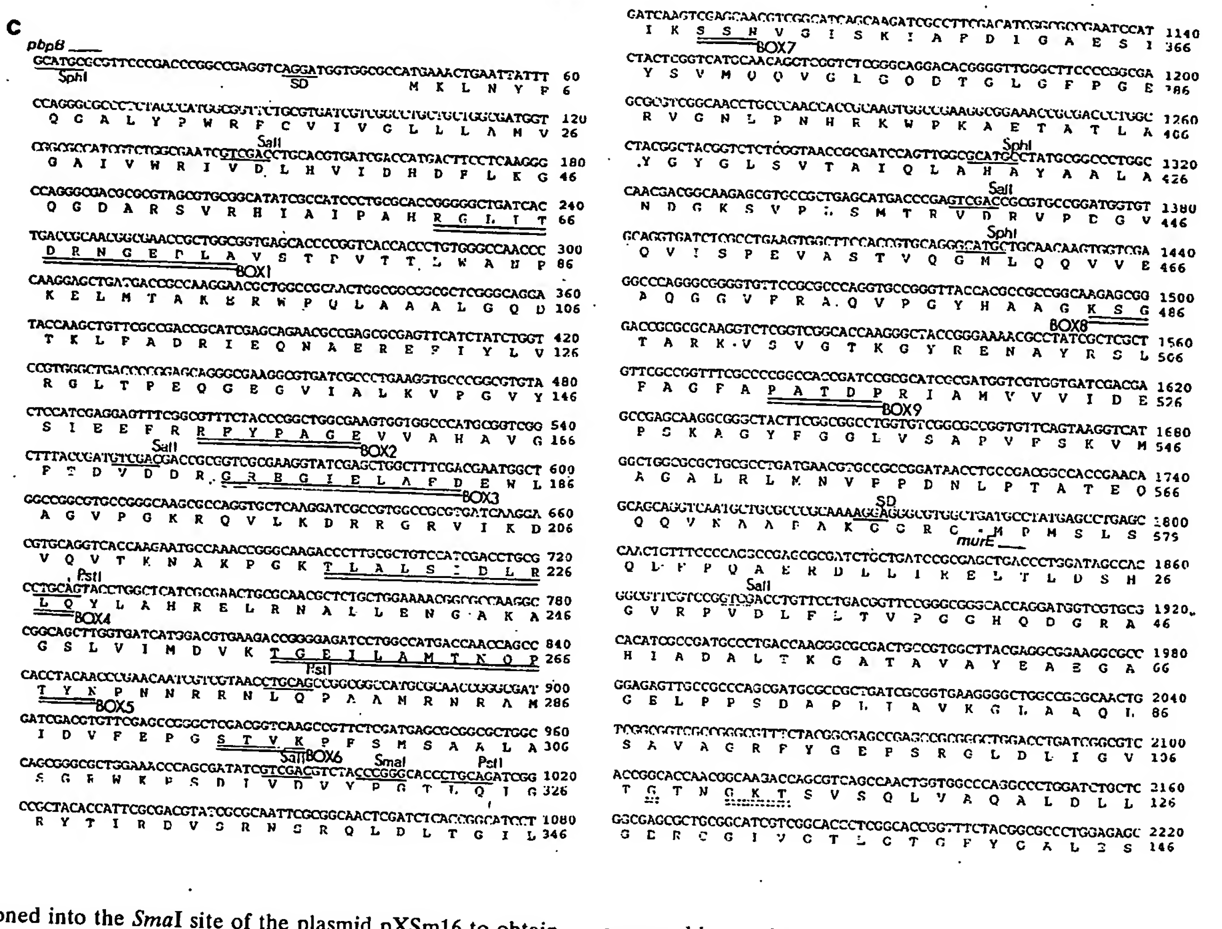

FIG. 1. (A) Physical map of the *P. aeruginosa pbpB* gene region. The light grey box indicates the location of the 580-bp PCR product. The dark grey and open boxes indicate the locations of ORF1 and ORF2, respectively. (B) Restriction enzyme map of the various *pbpB* subclones. The hatched boxes represent the PAO1 chromosomal DNA cloned into the vector pTZ19U. The arrows indicate the orientation of the *lac* promoter on the vector. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I; Sm, *Sma*I; Sp, *Sph*I; Xb, *Xba*I; X, *Xho*I; X/Sa, *Xho*I-*Sal*I. (C) Nucleotide sequence of the *pbpB* region from *P. aeruginosa* and deduced amino acid sequences of the two ORFs. The putative ribosome binding (Shine-Dalgarno [SD]) sites are underlined. The residues corresponding to the conserved boxes are indicated by double underlining. The domain A motifs of ATP-binding proteins are indicated by dashed underlining. Asterisk indicates stop codon.

cloned into the *Sma*I site of the plasmid pXSm16 to obtain pXLSH36 (Fig. 1B). This clone contained a 5.4-kb *Sph*I-*Xho*I fragment of the PAO1 chromosome DNA cloned into the vector pTZ19U. Both strands of a 2.7-kb *Sph*I-*Pst*I region were sequenced with an Applied Biosystems Inc. (Foster City, Calif.) model 373 automated fluorescence sequencer, after creation of nested deletions (8). Two translated open reading frames (ORFs) were found from this sequence (Fig. 1C). They were located at nucleotides 44 through 1783 and 1783 through 2757. The second ORF appeared incomplete at its 3′ end. The first ORF encoded a 579-amino-acid sequence with 45% identity and a total of 66% conserved amino acids compared with the *E. coli* PBP3. All nine conserved motifs of the high-molecular-weight PBPs (4, 15) were strongly conserved in this sequence. The *pbpB* gene was preceded by a Shine-Dalgarno sequence, AGGA, at positions 30 to 34, which matched reasonably the consensus ribosome binding site. Another initiation codon, ATG at positions 116 to 118, was believed unlikely to encode the first amino acid of the protein, since its upstream

GCCCGGCACACCACGCCGGACCCGCTCCCGCTGCAGGCCACGCCTGGCCACGCTGAAGCAG 2280
G R H T T P D P L A V Q A T L A T L K Q 166

GCCGGCGCCCGCGCGGTAGCGATGGAAGTGTCTTCCCACGGCCTCGACCAGGGCCGCGTG 2340
A G A R A V A H R V S S H G L D Q G R V 186

GCGGCGCTCGGCTTCGATATCGCGGTGTTCACCAATCTGTCCCGCGACCACCTCGACTAT 2400
A A L G F D I A V F T N L S R D H L D Y 206

CACGGTTCGATGGAAGCCTATGCCGCCGCCAAGGCCAAGCTGTTCGCCTGGCCGGACCTC 2460
H G S M E A Y A A A K A K L F A W P D L 226

SalI
CGCTGCCGGGTGATCAACCTGGACGACGATTTCGGCCGTCGACTGGCCGGCGAGGAGCAG 2520
R C R V I N L D D D F G R R L A G E E Q 246

GACTCGGAGCTGATCACCTACAGCCTCACCGACAGCTCCGCGTTCCTCTATTGCCGCGAA 2580
D S E L I T Y S L T D S S A F L Y C R E 266

GCGCGCTTCGGCGACGCCGGCATCGAGGCGGCGCTGGTCACTCCGCACGGCGAGGGCCTG 2640
A R F G D A G I E A A L V T P H G E G L 286

CTGCGCAGCCCGTTGCTCGGCCGCTTCAACCTGAGCAACCTGCTGGCGGCGGTCGGTGCG 2700
L R S P L L G R F N L S N L L A A V G A 306

PstI
TTGCTTGGCCTGGGTTATCCCCTGGGCGATATCCTCCGCACTTTGCCGCAGCTGCAG 2757
L L G L G Y P L G D I L R T L P Q L Q 325

sequence had no suitable ribosome binding site. This was further confirmed by the N-terminus amino acid sequence analysis (see below). The third position of codons constituted 85.8% of G+C content, typical of a high-G+C organism like *P. aeruginosa* (24). No promoter-like sequences were identified between the *Sph*I site and Shine-Dalgarno sequence.

The second ORF was incomplete and encoded 325 amino acids. This ORF started 2 nucleotides downstream from the end of the *pbpB* gene (Fig. 1C). Its sequence showed 43% identity and a total of 65% conserved amino acids compared with amino acids 1 to 337 of the *E. coli murE* gene product, uridine diphosphate-*N*-acetyl muramic acid-tripeptide synthetase. A putative Shine-Dalgarno sequence, AGGA, was located at positions 1768 to 1771. The third position of codons constituted 86.8% of G+C content. The putative ATP-binding sequences of the *murE* gene product were conserved. The *pbpB* and *murE* genes are also clustered in *E. coli* (12). The two genes were spaced 2 nucleotides apart in *P. aeruginosa*, whereas in *E. coli* the coding region for *murE* overlapped the end of the *pbpB* coding region by 11 bp. Interestingly, these genes mapped physically to the same region of the chromosome, *Spe*I fragment D2, *Dpn*I fragment I, as did the *ftsA-ftsZ-envA* genes (9a), suggesting that *P. aeruginosa*, like *E. coli*, may have a major cell division gene cluster.

Having observed that a novel PBP3 protein was not detected by the ^{3}H-penicillin G assay in *E. coli* DH5α containing plasmid pXLSH36, which had the *pbpB* gene cloned in the opposite orientation relative to the *lac* promoter in the vector pTZ19U, we then cloned the same 5.4-kb *Sph*I-*Xho*I fragment into the vector pTZ18U to create plasmid pXLBI3, which had the *pbpB* gene in the same orientation relative to the *lac* promoter. However, no novel protein was detected by ^{3}H-penicillin, probably because of a weak expression of the protein. Therefore, an efficient expression system (20) utilizing plasmid pT7-7 in combination with pGP1-2 was used. For the purpose of conveniently cloning the *P. aeruginosa pbpB* gene into the vector pT7-7 between the *Nde*I and *Bam*HI sites, PCR was utilized to amplify the *pbpB* gene. Primers for the PCR were 5′-TAAACATATGAAACTGAATTATTTCCAGGGCGCCCT-3′, which contained an *Nde*I recognition sequence and the sequence coding for the N terminus of PBP3, and 5′-AAAGGATCCTCAGCCACGCCCTCCTTTTGCGGGCGCA-3′, which contained the sequence coding for the C terminus of PBP3 followed by a stop codon and the sequence for a *Bam*HI recognition site. The PCR utilized Vent$_R$ DNA polymerase (New England Biolabs), which contains a 3′→5′ proofreading exonuclease activity resulting in

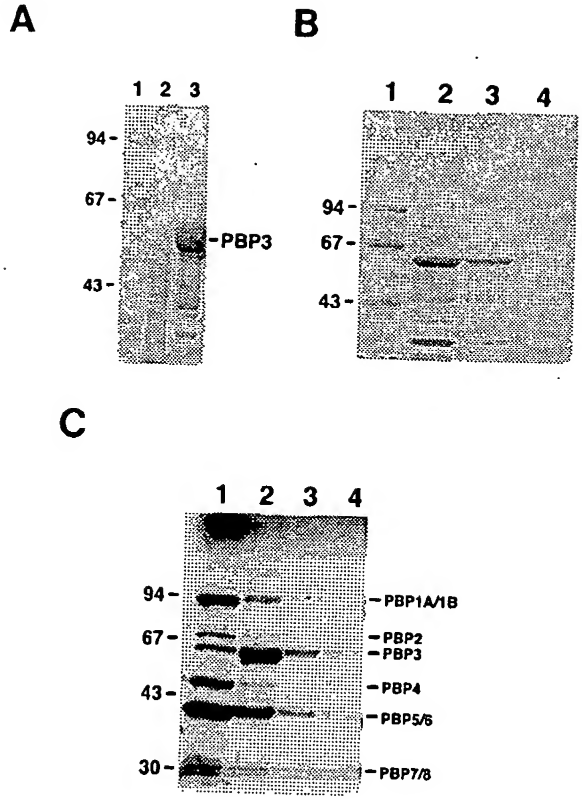

FIG. 2. (A and B) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of cell membrane proteins. *E. coli* BL21(DE3) cells grown in LB broth, supplemented with chloramphenicol for plasmid-containing bacteria, were harvested after induction with 1 mM IPTG for 3 h and washed in 10 mM $NaKHPO_4$ buffer, pH 7.0. After French Press (14,000 lb/in^2) treatment of cells and ultracentrifugation at 55,000 rpm (Beckman 70.1 Ti rotor) at 4°C for 1 h, membrane proteins were resuspended in the $NaKHPO_4$ buffer (10 mM, pH 7.0). (A) Lane 1, standard molecular mass markers; lane 2, *E. coli* BL21(DE3)/pBBR1MCS, the vector control for lane 3; lane 3, *E. coli* BL21(DE3)/pXL608, containing the cloned *P. aeruginosa pbpB* gene. (B) Lane 1, standard molecular mass markers; lane 2, *E. coli* BL21(DE3)/pXL608, 1/10 the amount of membrane proteins loaded in lane 3 of panel A; lane 3, *E. coli* BL21(DE3)/pXL608, one-fifth the amount of membrane proteins loaded in lane 2; lane 4, *E. coli* BL21(DE3)/pXL608, one-fifth the amount of membrane proteins loaded in lane 3. Numbers indicate molecular mass in kilodaltons. (C) Autoradiogram of cell membrane proteins after incubation with ^{3}H-penicillin G and separation by SDS-PAGE. PBPs were assayed by the method of Spratt (16). Membrane proteins were incubated with ^{3}H-penicillin G (3.7 μg/ml, 22 Ci/mol) at 23°C for 10 min. The reaction was stopped by the addition of an excess (1,000-fold) of nonradioactive penicillin G. The samples were separated by SDS-PAGE (8.5% acrylamide gel), and the resultant gel was autoradiographed on Kodak X-ray film for 33 days at −70°C, after being pretreated with 1 M sodium salicylate, pH 6.0, at 23°C for 30 min and dried at 80°C under a vacuum for 2 h (3). Lane 1, *E. coli* BL21(DE3)/pBBR1MCS, the same amount of membrane proteins as loaded in lane 2 of panel A and used as the vector control for lanes 2, 3, and 4; lane 2, *E. coli* BL21(DE3)/pXL608, the same amount of membrane proteins as loaded in lane 2 of panel B; lane 3, *E. coli* BL21(DE3)/pXL608, the same amount of membrane proteins as loaded in lane 3 of panel B; lane 4, *E. coli* BL21(DE3)/pXL608, the same amount of membrane proteins as loaded in lane 4 of panel B. Numbers indicate molecular mass in kilodaltons.

high fidelity of base incorporation. The 1,750-bp PCR product, corresponding to the size of the *pbpB* gene, was digested with *Nde*I and *Bam*HI and cloned into pT7-7 to make plasmid pXL706. This plasmid carried the *pbpB* gene behind the T7 promoter with the start codon of the *pbpB* gene 8 bp downstream from the ribosomal binding site on the vector. The protein was efficiently expressed in *E. coli* K38/pGP1-2 after T7 RNA polymerase was induced at 42°C. The amount of protein detected by ^{3}H-penicillin appeared to be somewhat less than expected given its abundance in the membrane protein sample (data not shown). This could possibly be due to incomplete removal of the β-lactamase produced by pT7-7. Therefore, an alternative approach utilizing a broad-host-range vector, pBBR1MCS, which carries a chloramphenicol resistance gene and T7 promoter (9), was used to express the *pbpB* gene product. A 1.75-kb *Xba*I-*Bam*HI fragment isolated from plasmid pXL706, which contained the ribosomal-binding-site sequence and the *pbpB* gene, was cloned in the vector pBBR1MCS behind the T7 promoter. After induction with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), the protein was expressed in *E. coli* BL21(DE3), which carries the T7 RNA polymerase gene under the control of the *lacUV5* promoter (19). The expressed protein had an apparent molecular weight of 60,000 (Fig. 2A and B). This *pbpB* gene product cofractionated with cytoplasmic membrane proteins. The ^{3}H-penicillin G assay showed that the *P. aeruginosa pbpB* gene product covalently bound penicillin (Fig. 2C).

The translated PBP3 protein containing 579 amino acids would have a calculated molecular mass of 63.69 kDa. However, the protein expressed from *E. coli* BL21(DE3)/pXL608 migrated with an apparent molecular mass of 60 kDa. N-terminal amino acid sequence analysis of this protein showed that it was identical to the translated sequence for the first 6 amino acids. This may reflect posttranslational C-terminal processing, as shown to occur with *E. coli* PBP3 before it is localized to the cytoplasmic membrane (13). The sequence at the N terminus was apparently not removed and did not appear to be characteristic of a typical signal peptide (22), nor did it contain a putative lipoprotein signal processing sequence as proposed for *E. coli* PBP3 (7).

All of the high-molecular-weight PBPs studied to date are known to be inner membrane proteins with their hydrophobic amino terminus anchored in the cytoplasmic membrane. Hydropathy analysis of the deduced amino acid sequence of *P. aeruginosa* PBP3 showed a profile similar to that of *E. coli* PBP3 (data not shown). Prediction of membrane-spanning segments by using the computer program PC gene indicated that there was only one transmembrane segment, stretching from residues 15 to 31 as the inner boundaries and residues 8 to 39 as the outer boundaries. The protein was thus predicted to be an integral inner membrane protein.

Nucleotide sequence accession number. The *pbpB* and *murE* sequence shown in Fig. 1C has been submitted to the EMBL data library and assigned the accession number X84053.

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REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1987. Current protocols in molecular biology. John Wiley & Sons, Inc., New York.
2. **Bell, A., and R. E. W. Hancock.** 1989. Outer membrane protein H1 of *Pseudomonas aeruginosa*: purification of the protein and cloning and nucleotide sequence of the gene. J. Bacteriol. 171:3211–3217.
3. **Chamberlain, J. P.** 1979. Fluorographic detection of radioactivity in polyacrylamide gels with the water-soluble fluor, sodium salicylate. Anal. Biochem. 98:132–135.
4. **Ghuysen, J.-M.** 1991. Serine β-lactamases and penicillin-binding proteins. Annu. Rev. Microbiol. 45:37–67.
5. **Godfrey, A. J., L. E. Bryan, and H. R. Rabin.** 1981. β-Lactam-resistant *Pseudomonas aeruginosa* with modified penicillin-binding proteins emerging during cystic fibrosis treatment. Antimicrob. Agents Chemother. 19:705–711.
6. **Hancock, R. E. W., and A. M. Carey.** 1979. Outer membrane of *Pseudomonas aeruginosa*: heat- and 2-mercaptoethanol-modifiable proteins. J. Bacteriol. 140:902–910.
7. **Hayashi, S., H. Hara, H. Suzuki, and Y. Hirota.** 1988. Lipid modification of *Escherichia coli* penicillin-binding protein 3. J. Bacteriol. 170:5392–5395.
8. **Huang, H., R. Siehnel, F. Bellido, E. Rawling, and R. E. W. Hancock.** 1992. Analysis of two gene regions involved in the expression of the imipenem-specific outer membrane porin proteins oprD of *Pseudomonas aeruginosa*. FEMS Microbiol. Lett. 97:267–274.
9. **Kovach, M. E., R. W. Phillips, P. H. Elzer, R. M. Roop II, and K. M. Peterson.** 1994. pBBR1MCS: a broad-host-range cloning vector. BioTechniques 16:800–802.

9a. **Liao, X., I. Charlebois, C. Ouellet, M.-J. Morency, K. Dewar, J. Lightfoot, J. Foster, R. Siehnel, H. Schweizer, J. S. Lam, R. E. W. Hancock, and R. C. Levesque.** Physical mapping of 32 genetic markers on the Pseudomonas aeruginosa PAO1 chromosome. Submitted for publication.

10. **Maejima, T., M. Inoue, and S. Mitsuhashi.** 1991. In vitro antibacterial activity of KP-736, a new cephem antibiotic. Antimicrob. Agents Chemother. 35:104–110.
11. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
12. **Michaud, C., C. Parquet, B. Flouret, D. Blanot, and J. Van Heijienoort.** 1990. Revised interpretation of the sequence containing the *murE* gene encoding the UDP-N-acetylmuramyl-tripeptide synthetase of *Escherichia coli*. Biochem. J. 269:277–280.
13. **Nagasawa, H., Y. Sakagami, A. Suzuki, H. Suzuki, H. Hara, and Y. Hirota.** 1989. Determination of the cleavage site involved in C-terminal processing of penicillin-binding protein 3 of *Escherichia coli*. J. Bacteriol. 171:5890–5893.
14. **Noguchi, H., M. Matsuhashi, and S. Mitshhashi.** 1979. Comparative studies of penicillin-binding proteins in *Pseudomonas aeruginosa* and *E. coli*. Eur. J. Biochem. 100:41–49.
15. **Piras, G., G. Raze, A. El Karroubi, D. Hastir, S. Englebert, J. Coyette, and J.-M. Ghuysen.** 1993. Cloning and sequencing of the low-affinity penicillin-binding protein 3r-encoding gene of *Enterococcus hirae* S185: molecular design and structural organization of the protein. J. Bacteriol. 175:2844–2852.
16. **Spratt, B. G.** 1977. Properties of the penicillin-binding proteins of *Escherichia coli*. Eur. J. Biochem. 72:341–352.
17. **Spratt, B. G.** 1983. Penicillin-binding proteins and the future of β-lactam antibiotics. J. Gen. Microbiol. 129:1247–1260.
18. **Sprat, B. G., and K. D. Cromie.** 1988. Penicillin-binding proteins of Gram negative bacteria. Rev. Infect. Dis. 10:699–711.
19. **Studier, F. W., and B. A. Loffat.** 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. 189:113–130.
20. **Tabor, S., and C. C. Richardson.** 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA 82:1074–1078.
21. **Tipper, D. J., and J. L. Strominger.** 1965. Mechanism of function of penicillins: a proposal based on their structural similarity to acyl-D-alanyl-D-alanine. Proc. Natl. Acad. Sci. USA 54:1133–1141.
22. **von Heijine, G.** 1990. The signal peptide. J. Membr. Biol. 115:195–201.
23. **Watanabe, N.-A., K. Katsu, M. Moriyama, and K. Kitoh.** 1988. In vitro evaluation of E1040, a new cephalosporin with potent antipseudomonal activity. Antimicrob. Agents Chemother. 32:693–701.
24. **West, S. E. H., and B. H. Iglewski.** 1988. Codon usage in *Pseudomonas aeruginosa*. Nucleic Acids Res. 16:9323–9335.